The role of factor Va in the formation and function of the prothrombinase complex

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CONTENTS

ABBREVIATIONS

DEAE	diethylaminoethyl
DFP	diisopropyl fluorophosphate
DOPA	1,2-dioleoyl-sn-glycero-3-phosphatidic acid
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPS	1,2-dioleoyl-sn-glycero-3-phosphoserine
DMPS	1,2-dimyristoyl-sn-glycero-3-phosphoserine
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPH	1,6 diphenyl-1,3,5 hexatriene
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
M_r	relative molecular weight
SDS	sodiumdodecyl sulfate
QAE	quaternary amino ethyl or [diethyl(2-hydroxyporpyl) amino]ethyl
RVV-V	factor V activator purified from Russell's Viper Venom
RVV-X	factor X activator purified from Russell's Viper Venom
SP	sulfopropyl
S2222	N-benzoyl-L-isoleucyl-L-glutamyl-piperidyl-glycyl--L-arginine-p-nitroanilide
S2238	D-phynylanalyl-L-pipecolyl-L-arginine-p-nitroanilide
S2337	N-benzoyl-L-isoleucyl-L-glutamyl-piperidyl-glycyl--L-arginine-p-nitroanilide
Tris	tris (hydroxylmethyl)aminomethane
Va HC	factor Va heavy chain, Mr=94,000
Va LC	factor Va light chain, Mr=80,000

CHAPTER I

INTRODUCTION

Defects in the process of blood clotting as manifested by, for example, bleeding tendency or thrombosis has, for a long time, given sufficient reason to search for possible corrections of these abberations. Over the years, research into the macroscopic phenomena of blood clotting, has led to the step by step discovery of the components responsible for these phenomena; the functions and relationships between these components are still a matter of investigation.

Biochemical studies of the phenomena utilizing advanced techniques, improved isolation procedures of blood clotting factors and the use of synthetic substrates and well defined phospholipids, have allowed a number of molecular details to become clear.

In the next section, some features of the blood clotting process are outlined in order to familiarize the reader with the subject of the studies described in this thesis. For detailed reviews, see Suttie & Jackson (1977), Davie et al. (1979) and Jackson & Nemerson (1980).

The process of blood clotting

The regulation of the blood clotting process is controlled by interactions between a great number of components. These components, namely proteins, calcium and complex phospholipid stuctures (e.g. blood platelets membranes) are circulating in the blood and/or reside in the vessel walls. A number of the proteins have been found to be serine proteases which are present in their unactivated (zymogen) forms.

The blood clotting process consists of a series of enzyme activations and in each step of this sequence, the activated zymogen activates another zymogen to an active protease. The linear sequence of activations of zymogens was first represented as a cascaded reaction system (Macfarlane, 1964).

However, evidence was provided that complexes that consist of an enzyme together with a non-enzymatic protein cofactor and phospholipid, formed the

catalytic units in the process of blood clotting (Hemker et al., 1967). The molecular organization of such a complex is shown in Figure 1. The complex cascaded reaction system evolved into a series of discrete and simpler stages, i.e. a staged cascade system. The modern concept of the blood clotting process is depicted in Figure 2. Interesting features are the by-pass routes, e.g. the activation of factor IX by factor VIIa, and the feedback mechanisms, e.g. activation of factor V and factor VIII by thrombin.

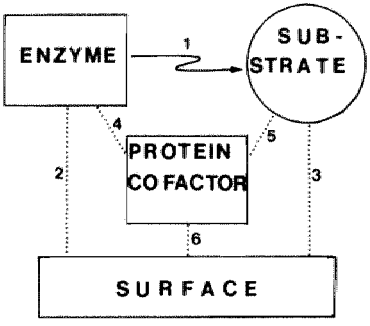

FIGURE 1: Pairewise interactions between the components participating in a stage of the blood clotting cascade. When the enzyme and the substrate are vitamin K-related proteins [prothrombin, X(Xa), IX(IXa) and VII(VIIa)], the interactions presented by tie lines 2 and 3 involve Ca^{++}, and the surface is phospholipid. Interaction of the protein cofactors [Va, VIII(VIIIa) and tissue factor (TF)] (tie line 6), do not appear to directly involve Ca^{++}.

[taken from Jackson & Nemerson, 1980]

The components of the prothrombinase complex

As yet, prothrombinase, the subject of our investigations, is the best documented complex enzyme system involved in blood clotting. The complex consists of the serine protease factor Xa, the protein cofactor factor Va, calcium ions and phospholipid. The complex converts prothrombin into the serine protease thrombin.

Factor Xa. Factor Xa is generated by limited proteolysis of factor X (M_r=55,000), a glycoprotein consisting of a 48,000 dalton polypeptide linked together by disulfide bridges to a 17,000 dalton polypeptide. The NH_2-terminal region of the 17,000 dalton peptide contains 12 γ-carboxyglutamyl residues (Enfield et al., 1975). The γ-carboxyglutamyl residues are synthesized

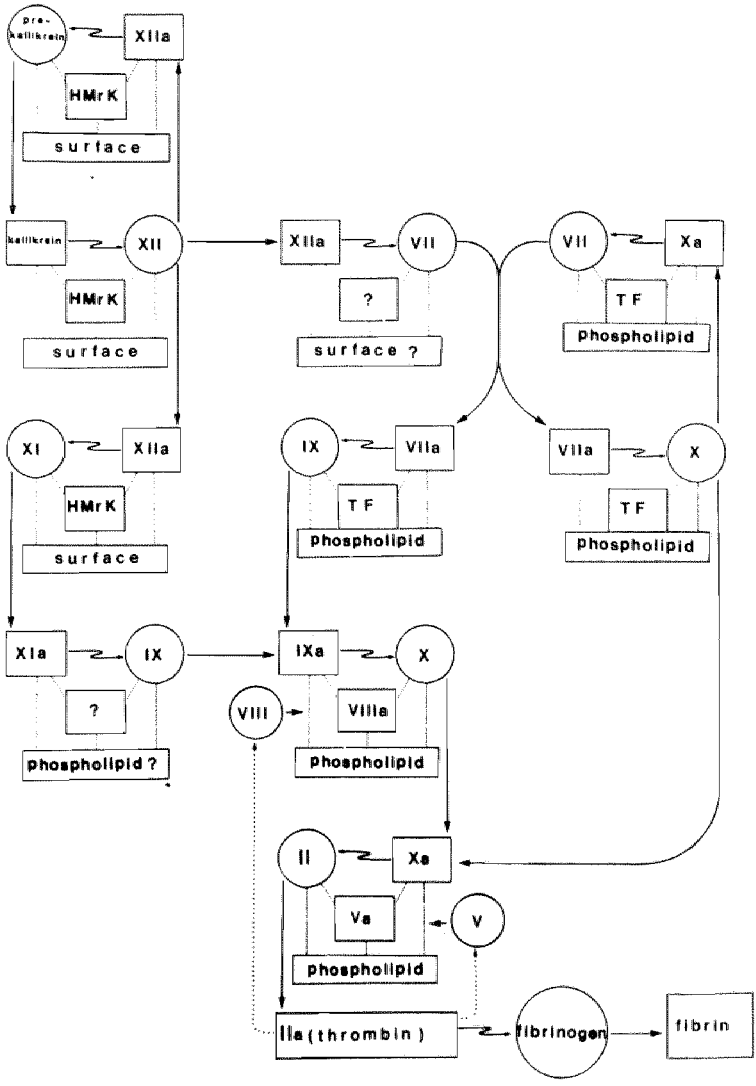

FIGURE 2: A staged cascade representation of the blood clotting system.

The numbers refer to the respective clotting factors , HMrK is high-molecularweight kininogen , TF is the protein component of the tissue factor.

from glutamyl residues in a post-ribosomal precursor by a vitamin K-dependent carboxylase [see Suttie & Jackson (1977) for a review]. These modified glutamyl residues are involved in the Ca^{++}-mediated binding to phospholipid (Nelsestuen & Lim, 1977; Dombrose et al., 1979). The 48,000 dalton peptide of factor X contains the active site that becomes exposed after splitting off of a polypeptide of 51 amino acid residues at the NH_2-terminus.

Prothrombin. Prothrombin is a single chain polypeptide (M_r=72,000). The NH_2-terminal region contains 10 γ-carboxyglutamyl residues (Magnusson et al., 1974). Therefore, prothrombin, like factor X, is called a vitamin K-dependent protein.

Numerous studies [see Jackson & Nemerson (1980) for a review] on the conversion of prothrombin to thrombin by factor Xa alone or by the prothrombinase complex, have revealed the mechanism of activation of prothrombin as depicted in Figure 3. Fragment 1 contains the phospholipid-binding region of prothrombin, fragment 2 probably interacts with factor Va and the B-chain of thrombin contains the active serine residue.

Phospholipid. The procoagulant activity of artificially prepared membranes appears to depend on the chemical composition and the physical state of the membrane. From the pioneering work of Bangham (1961) and Papahadjopoulos (1964), it became clear that negatively charged phospholipid is involved in prothrombin activation. Among the negatively charged phospholipids, phosphatidylserine seems the most potent. However, they can also be substituted by phosphatidylinositol (Zolton & Seegers, 1974) or phosphatidylglycerol (Esmon et al., 1974). Mixing of the negative phospholipid with neutral lipids appears necessary (Daemen et al., 1965; Bull et al., 1972). Futhermore, it was found that the fatty acid chain length and the degree of saturation influence the procoagulant activity (Sterzing & Barton, 1973; Barton & Findly, 1977).

It was observed that phospholipid mixtures below the phase transistion temperature exhibit little procoagulant activity and liquifying of the membrane by adding cholesterol (Sterzing & Barton, 1973) or heating above the transition temperature (Tans et al., 1979), greatly increased the procoagulant activity. Optimal activity was found for biphasic lipid systems (Barton & Findly, 1977). However, Tans et al. (1979) reported that vesicles composed of phospholipids that have a poor miscibility have low procoagulant activity, even above the transition temperature.

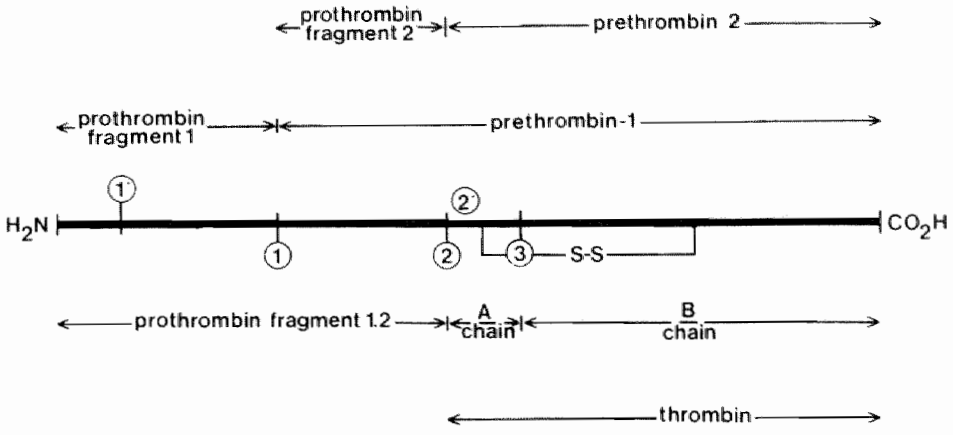

FIGURE 3: Schematic stucture for prothrombin. The three peptide bonds that are cleaved in bovine prothrombin during or as a result of prothrombin activation are designed by 1 , 2 ,and 3 . Another bond, 1 , can be cleaved in both bovine and human prothrombin. However, neither the agent nor the precise location has been determined. The nomenclature system for prothrombin proteolysis products, proposed by a Task Force of the International Committee on Thrombosis and Haemostasis is designated as follows: if bond 1 is cleaved in prothrombin, two products are formed: prothrombin fragment 1 and prethrombin 1. Bovine prothrombin fragment 1 consisits of the region from the amino terminus to bond 1 (Arg 156 - Ser 157); prethrombin 1 is the polypeptide from bond 1 to the carbonyl terminus of prothrombin. By convention, prethrombin implies that the amino sequence ultimately giving rise to thrombin is contained in the polypeptide; fragment implies that the amino acid sequence of the polypeptide is not finally associated with thrombin. Cleavage of bond 2 (Arg 274 - Thr 275) gives rise to prothrombin fragment 1.2 and prethrombin 2. Cleavage of bond 1 in prothrombin fragment 1.2 yields prothrombin fragment 1 and prothrombin fragment 2. Cleavage of bond 3 (Arg 323 - Ile 324) in prethrombin 2 yields thrombin, a disulfide-linked, two-chain enzyme in which the polypeptide chain between bonds 2 and 3 is designated the A-chain and the polypeptide chain between bond 3 and the carboxyl terminus is designated the B-chain.
[taken from Suttie & Jackson, 1977]

In vivo, the procoagulant surface is provided by the membrane of blood platelet. The lipid composition of the platelet membrane has been well documented. It was shown that the different lipid classes are asymetrically distributed over the membrane leaflets. Neutral phospholipid, e.g. sphingomyelin and phosphatidylcholine, are mainly found in the outer leaflet, while phosphatidylethanolamine and negatively charged phospholipids, e.g. phosphatidylserine, are almost exclusively located in the inner leaflet of unactivated platelets (Zwaal, 1978). After stimulation of the platelets by a combination of thrombin and collagen, the phosphatidylserine and phosphatidylethanolamine content of the outer leaflet greatly increases, concomitant with the appearance of procoagulant (prothrombinase) activity (Bevers et al., 1982).

Biomembranes consist of phospholipid bilayers and structural or functional protein components. These proteins are responsible for a number of physical properties of the membranes (Van Deenen, 1981; Zwaal & Hemker, 1982). Therefore, caution has to be taken as to the interpretations of studies utilizing artificially prepared membranes for in vivo situations.

Factor Va. Factor V was discovered by Nolf (1908) and rediscovered by Quick (1943), who described a labile protein required for the activation of prothrombin. Ware & Seegers (1948) and Owren (1953) were the first to recognize that this instable protein (proaccelerine) could be converted in a more active form (accelerine). Since factor V was difficult to purify in an undegraded form without contaminating proteins, the molecular details of the process of activation of factor V have not been established until recently.

Methods of the purification of a homogeneous and undegraded factor V from bovine plasma were reported by Esmon (1979) and Nesheim et al. (1979). Highly purified human factor V was isolated by Kane et al. (1981) and Suzuki et al. (1982). Factor V from bovine or human origin is a single chain glycoprotein with M_r=330,000.

Factor V possess only 0.25% of the activity of thrombin-activated factor V (Nesheim et al., 1979). It is not known whether this is due to its intrinsic activity or is caused by a contamination with activated factor V. Since factor V activity is measured in an assay that requires the measurement of the rate of thrombin formation, precise determinations of the so-called activation quotients are extremely difficult.

The activation of highly purified single-chain bovine and human factor V by thrombin has been extensively studied. Table I lists the components seen during the activation of factor V by thrombin. Since the proteolysis products are poorly characterized, disagreement between the reports on the activation mechanism of factor V still exists. However, at the moment, the schematic model for the thrombin-catalyzed activation of human factor V, as given by Suzuki et al. (1982, 1983) is the most plausible one (Figure 4).

From our data (Lindhout et al., 1982) it is reasonable to conclude that the activation of bovine factor V does not differ significantly from that of human factor V. Like the bovine factor Va, the human factor Va molecule consists of two noncovalently linked polypeptide chains, factor Va LC (M_r=80,000 or M_r=71,000-74,000) and factor Va HC (M_r=94,000 or M_r=105,000).

Table I: Components Seen During the Activation of Human and Bovine Factor V by Thrombin[a]

	Human factor V[b]	Human factor V[c]	Bovine factor V[d]	Bovine factor V[e]	Bovine factor V[f]
Factor V	335	330	330	350	290
Activation intermediates	295	280	g	280	g
	248	220	205	220	220
	g	g	150	g	g
Final products	150	150	g	150	140
	121	105[h]	94[h]	94[h]	110[h]
	g	71	92	65	g
	91-95[h]	74-71[h]	74-71	80[h]	73[h]
	g	g	31	g	g

[a] Apparent molecular weights expressed x 10^{-3}; [b] Kane & Majerus (1981); [c] Suzuki et al. (1982); [d] Nesheim & Mann (1979); [e] Lindhout et al. (1982); [f] Esmon (1979); [g] Not found; [h] Peptides that constitute factor Va according to the authors

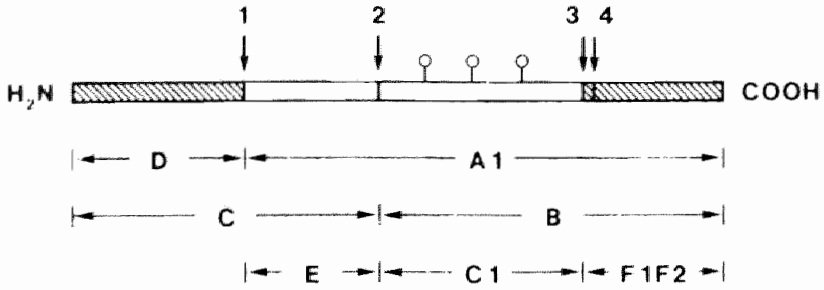

FIGURE 4: Schematic diagram of human factor V molecule. The peptide bonds cleaved by thrombin on activation of factor V (M_r=330,000) to factor Va are indicated by the arrows. The shaded parts [fragments D (M_r=105,000) and F1F2 (M_r=71-74,000)] are held together by noncovalent bonds in the presence of calcium ions and constitute the factor Va molecule. The two activation fragments E and C1, apparent M_r=71,000 and 150,000, respectively, are derived from the center of the polypeptide chain. O denotes that fragment C1 is very rich in carbohydrate. Fragments A1 and B are activation intermediates. Fragment C is a fragment observed in partly degraded factor V. Fragment A2 (not shown) is formed on RVV-V-catalyzed cleavage of the peptide bonds between fragments C1 and F1F2. Fragments D and F1F2 appear to correspond to the heavy and light chains, respectively, in Esmon's (1979) nomenclature for bovine factor Va.

[taken from Suzuki et al., 1983]

Calcium ions are required to stabilize the two-subunit structure of factor Va.

As a consequence of activation by thrombin, factor V acquires prothrombin and factor Xa binding-sites (Esmon et al., 1973). However, differences in affinities of factor V and factor Va for prothrombin and factor Xa have yet to be quantitated. Moreover, knowledge is lacking as to the interactions of the individual subunits of factor Va with prothrombin and factor Xa. Activation of factor V is not required in order to expose the phospholipid-binding sites on the factor V molecule. The phospholipid-binding properties of factor V are about the same as those of factor Va. We demonstrated that factor Va HC is bound to phospholipid by means of factor Va LC (van de Waart et al., 1983).

Phospholipid-binding properties of prothrombin, factor Xa and factor Va

Molecular models for the Ca^{++}-mediated association of the vitamin K-dependent proteins factor Xa and prothrombin with phospholipid have been proposed in which Ca^{++} "bridges" link the carbonyl groups of the γ-carboxy-glutamyl residues of the protein to the phosphate groups of negatively charged phospholipids. Dombrose et al. (1979) suggested a significant electrostatic contribution to the binding of protein to phospholipid. However, Resnick & Nelsestuen (1980) found evidence that ionic bridging between the protein and phospholipid is not important. They proposed a chelation model where the two interacting species have no net negative charge; ligands on the protein complete the coordination sphere of phospholipid-bound calcium and vice versa.

The dissociation constants of prothrombin- and factor Xa-phospholipid complexes increase with a decrease in negatively charged phospholipid content of the membrane. Clustering of these phospholipids around the protein is accompanied by an increase in positive free energy, which may be the major factor for the dependency of the dissociation constant on acidic lipid content (Nelsestuen & Broderius, 1977; Mayer & Nelsestuen, 1981). Approximately 5 and 10 phosphatidylserine molecules are involved in the phospholipid-binding of factor Xa and prothrombin, respectively. The dissociation constants for both proteins are 2×10^{-7} M at saturating Ca^{++} concentrations and phosphatidylserine content of the vesicles of >20% (w/w).

Factor Va-phospholipid interaction was assumed to be hydrophobic in nature (Kahn & Hemker, 1969; Bloom et al., 1979). Therefore, the requirement for negatively charged phospholipid could not be explained. Evidence for an electrostatic interaction, where positively charged amino acid residues interact with the phosphate groups of phospholipid, was given by Pusey et al. (1982) and Van de Waart et al. (1983). Conflicting data as to the dissociation constant of factor V(a)-phospholipid interaction were reported, i.e. 10^{-7} M (Bloom et al., 1979), 10^{-8} M (Van de Waart et al., 1983) and 10^{-11} M (Pusey et al., 1982). Platelets bind factor Va with a dissociation constant of 10^{-10} M (Tracy et al., 1979).

The role of factor Va in the assembly and function of the prothrombinase complex

Conceptual models of the prothrombinase complex have been presented (Figure 5). However, many questions have to be answered about the mechanism responsible for the enhancement of the rate of prothrombin activation by factor Va and phospholipid. Determinations of the relative rates of activation under conditions where phospholipid or factor Va were added to a mixture of prothrombin, factor Xa and Ca^{++}, did not allow conclusions about the way these components are involved in the process of prothrombin activation (Jobin & Esnouf, 1967; Esmon et al., 1974; Nesheim et al., 1979).

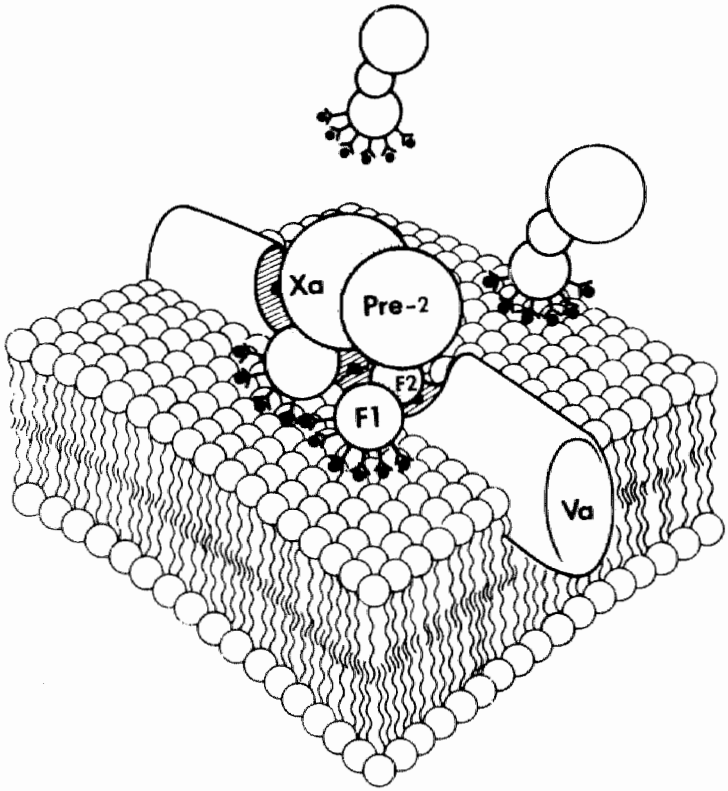

FIGURE 5: Model of prothrombinase. The enzyme prothrombinase is depicted as a stoichiometric (1:1), Ca^{++}-dependent complex of phospholipid-bound factor Va and factor Xa.
[taken from Nesheim et al., 1980]

Rosing et al. (1980) were able to reveal the effects of phospholipid and factor Va on the kinetic parameters of prothrombin activation (Table II). Phospholipid decreased the K_m for prothrombin, while factor Va increased the V_{max} of thrombin formation. They concluded from their results that a complex between factor Xa and factor Va is the catalytic unit in prothrombin activation. They proposed that factor Va prevents the dissociation of prethrombin 2 from the prothrombinase complex.

Table II: Kinetic Constants of Prothrombin Activation

Enzyme	Substrate	Accessory components	K_m (μM)	k_{cat} (s^{-1})
fXa	prothrombin	Ca^{2+}	84	0.011
fXa	prothrombin	Ca^{2+}, fVa	34	6.22
fXa	prothrombin	Ca^{2+}, phospholipid	0.06	0.038
fXa	prothrombin	Ca^{2+}, phospholipid, fVa	0.2	32.0

Factor Va-factor Xa interaction at a phospholipid surface. Studies on the interaction between factor Va and factor Xa in free solution, as probed by prothrombin activation kinetics, showed the formation of a stoichiometric (1:1) complex with a dissociation constant of 3×10^{-9} M (Lindhout et al., 1982). Because both factor Xa and factor Va bind to phospholipid, it is easy to conceive that phospholipid promotes the complex formation, as demonstrated by a decrease in dissociation constant. Several studies, utilizing different techniques, revealed that factor Xa and factor Va form a stoichiometric (1:1) complex at the phospholipid surface. The dissociation constant of the complex in the presence of phospholipid was found to be 10^{-11} M. As the affinities of factor Xa and factor Va depend on the phosphatidylserine content of the membrane, the dissociation constant of the complex-phospholipid interaction was also found to be dependend on the phosphatidylserine content (Nesheim et al., 1979; Nesheim et al., 1981; Lindhout et al., 1982).

Factor Xa-factor Va interaction at the platelet surface. Evidence that human platelets also promote the interaction between factor Xa and factor Va was provided by Miletich et al. (1978). The same was reported for bovine platelets by Dahlbäck & Stenflo (1978) and Tracy et al. (1981). A stoichiometry of 1:1 and a dissociation constant of 10^{-10} M were reported. However, the binding interaction appears to be more complicated than that at an artificially prepared membrane. Questions are raised as to the necessity of platelet activation by thrombin in order to obtain a functional binding surface. This activation process is thought to be necessary for the exposure of phosphatidylserine molecules, required for the binding of both factor Xa and factor Va, at the outer surface of the platelets (Zwaal & Hemker, 1982).

Interaction between prothrombin and factor Xa-factor Va. Esmon et al. (1974) found no effect of factor Va on the conversion of prethrombin 2 by prothrombinase. They suggested that prothrombin fragment 2 contains the factor Va binding region of prothrombin. From more detailed kinetic studies, it was concluded that factor Va is also essential to the proper formation of the enzyme-substrate complex (Rosing et al., 1980). Evidence for an interaction between factor Va and prothrombin was provided by Esmon et al. (1973) utilizing immobilized prothrombin. Direct evidence for the interaction between factor Va and prothrombin is provided in this thesis utilizing an equilibrium binding technique.

Regulation of prothrombinase activity by activated protein C

With the discovery of the vitamin K-dependent protein C by Stenflo (1976), an additional sequence of reactions could be added to the process of blood clotting. Human protein C, M_r=62,000, consists of two polypeptide chains with M_r=21,000 and M_r=41,000, held together by disulfide bridges. The light-chain contains 11 γ-carboxyglutamyl residues (Kisiel, 1979). Bovine protein C does not differ significantly from human protein C (Kisiel et al., 1976).

In vivo activation of protein C occurs by a complex enzyme system. Thrombin is the protease, thrombomodulin acts as a cofactor and the surface is provided by the vessel wall (Esmon et al., 1982). Salem et al. (1983) reported that factor Va and the light chain of factor Va can serve also as the cofactor in activation of protein C by thrombin.

Activated protein C exhibits anti-coagulant activity by selective proteolysis of factor Va (Canfield et al., 1978; Walker et al., 1979) and factor VIII:C (Vehar & Davie, 1980). Activated protein C is also involved in the regulation of fibrinolysis (Comp & Esmon, 1981).

The inactivation of human and bovine factor Va by activated protein C occurs by a complex enzyme system, where phospholipid (platelets) provide the surface. Protein S, a vitamin K-dependent protein, is likely the cofactor of the reaction (Stenflo & Johnson, 1979; Stenflo & Dahlbäck, 1980; Walker, 1981).

Upon inactivation of factor Va, several peptide bonds are cleaved. The heavy chain of factor Va appears to be more susceptible for activated protein C as compared to the light chain of factor Va. The inactivation of factor Va seems to be the result of the cleavage of factor Va heavy chain (Walker et al., 1979; Tracy et al., 1983; Suzuki et al., 1983; this thesis). An interesting observation is that in the presence of phospholipid, factor Xa protects factor Va from inactivation by activated protein C (Walker et al., 1979; Nesheim et al., 1982; Suzuki et al., 1983).

The present study

The role of factor Va in the kinetics of prothrombinase has recently been studied extensively. However, the validity of the molecular models emerging from these studies is limited by the lack of quantative binding data. In our study, we therefore focussed on the characterization of the binding interactions between factor Va and the other components of the prothrombinase complex.

In chapter II we described our investigations into the interaction between factor Xa and factor Va. Attempts were made to localize the factor Xa binding site on factor Va.

A direct method for the determination of binding paramaters of protein-phospholipid interaction is described in chapter III. By this technique we established the nature of factor Va-phospholipid interaction. The effect of factor Va on membrane structures was studied by the technique of fluorescence depolarization (chapter IV).

The interactions between factor Va and factor Xa, as well as factor X and prothrombin, at a phospholipid surface are described in chapter V.

The physiological significance of factor Va-prothrombin interaction was inferred from kinetic studies on prothrombin activation (chapter VI).

The effect of phospholipid on the rate of inactivation of factor Va by activated protein C and the binding properties of the proteolytic altered factor Va subunits are described in chapter VII.

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CHAPTER II

FACTOR Va - FACTOR Xa INTERACTION. EFFECTS OF PHOSPHOLIPID VESICLES OF VARYING COMPOSITION.

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The interaction between factor Xa and factor Va was investigated both in solution and in the presence of phospholipid vesicles with varying contents of phosphatidylserine. The binding parameters were inferred from the kinetics of prothrombin activation. Factor Xa and factor Va form in solution an equimolar complex with a dissociation constant of 3.3×10^{-9} M. Phospholipid vesicles promote the formation of the factor Xa-Va complex. The K_d of complex formation is dependent both of the phospholipid concentration and the composition of the phospholipid vesicle. For the interaction between factor Xa and factor Va in the presence of phospholipid vesicles containing 40 mole % dioleoylphosphatidylserine (DOPS) and 60 mole % dioleoylphosphatidylcholine (DOPC), the K_d increases linear with increasing phospholipid concentration. In the presence of 10 μM phospholipid (DOPS/DOPC, 40/60; mol/mol) the $K_d = 3 \times 10^{-11}$ M. When the mole percentage DOPS in the phospholipid vesicles is lowered from 20 to 5 mole % there is a gradual increase of the K_d. In the presence of 10 μM phospholipid vesicles containing 5 mole % DOPS and 95 mole % DOPC the $K_d = 2.8 \times 10^{-10}$ M. The K_d measured in the presence of phospholipid vesicles containing 5 mole % DOPS and 95 mole % DOPC is independent of the phospholipid concentration. Two models are discussed, that can quantitatively explain the effect of phospholipid vesicles on the complex formation between factor Xa and factor Va. Studies on the effect of the polypeptides with $M_r = 80,000$ and $M_r = 94,000$ of which factor Va is composed on the K_d of the factor Xa.Va complex suggest that factor Xa binding to factor Va requires a Ca^{2+} mediated interaction between the two polypeptides.

INTRODUCTION

The activation of blood coagulation factor II (prothrombin) into thrombin, catalyzed by the serine protease factor Xa, requires at physiological conditions the presence of the non-enzymatic components phospholipid, Ca^{2+} ions and factor Va. It has been shown, that these so-called accessory components augment the rate of prothrombin activation and that a prothrombinase complex composed of factor Xa, negatively charged phospholipid, Ca^{2+} and factor Va is the most efficient in catalyzing prothrombin activation (Suttie & Jackson, 1977).

Kinetic studies on prothrombin activation by prothrombinase complexes of different composition (factor Xa either in the absence or presence of phospholipid, Ca^{2+} and/or factor Va) reveal that phospholipids cause a profound decrease of the K_m for prothrombin, while factor Va causes a drastic increase of the V_{max} of thrombin formation (Rosing et al., 1980). The changes of the kinetic parameters caused by the accessory components are consistent with a model in which phospholipid provide a surface upon which factor Xa, factor Va and prothrombin interact at increased local concentrations and factor Va changes the catalytic capacity of factor Xa.

Another important function of factor Va in haemostasis was discovered by Miletich et al. (1978). Their experiments suggest that factor Va is part of a highly specific factor Xa binding site on human blood platelets. The same tight binding site for factor Xa is also present at the surface of bovine blood platelets (Dahlbäck et al., 1978). Recently, Tracy et al. (1981) demonstrated that factor Xa and factor Va interact stoichiometrically at the platelet surface and that factor Xa binds to platelet-bound factor Va with an apparent dissociation constant of 6×10^{-10} M. Similar binding parameters were obtained in a phospholipid model system (Nesheim et al., 1979b).

The precise mode of action of phospholipid in promoting the interaction between factor Xa and factor Va is not known. In fact, it is not unequivocally demonstrated that factor Xa.Va complex formation at the platelet surface is the result of protein-phospholipid interaction per se. While Bevers

et al. (1982) reported that triggering of platelets with collagen plus thrombin results in the exposure of phosphatidylserine molecules at the platelet outer surface with a concomitant increase in prothrombinase activity, Kane et al. (1980) and Tracy et al. (1981) observed that platelet activation is not required for the interaction of factor Xa with factor Va at the platelet surface.

The purpose of the experiments described in this paper is to assess the contribution of protein-protein and protein-phospholipid interactions to the specificity and high affinity of the binding of factor Xa to phospholipid-bound factor Va. Therefore, we studied the interaction of factor Xa with factor Va in the absence and in the presence of phospholipid vesicles with a varying content of phosphatidylserine.

In a first attempt to reveal the structural requirements of the factor Va molecule for binding factor Xa, we also studied the interaction between factor Xa and isolated subunits with molecular weights of 80,000 and 94,000 of which factor Va is composed (Esmon, 1979).

MATERIALS AND METHODS

S-2238 was purchased from AB Kabi Diagnostica. Benzamidine and DFP were obtained from Sigma. All reagents used were of the highest grade commercially available.

Purification of Proteins

Bovine factors X_1 and X_2 were prepared according to Fujikawa et al. (1972a). RVV-X was purified from the crude venom as described by Schiffman et al. (1969). Bovine factor Xa was prepared from bovine factor X_1 using RVV-X according to the method of Fujikawa et al. (1972b). Bovine prothrombin and thrombin were purified as described earlier (Rosing et al., 1980). Prethrombin 1 was purified according to the method of Owen et al. (1974). Decarboxyprothrombin was a kind gift of Dr. Vermeer.

Bovine factor V was purified by a modification of the method of Esmon (1979). Bovine blood was obtained by venepuncture of cows treated with phen-

procoumon (average levels of the factors II and X were 20% and 5% respectively). The blood was collected in 1-liter plastic bottles, containing 100 ml of 100 mM sodium oxalate, 100 mM benzamidine, 20,000 units of heparin and 0.2 mg/ml of soybean trypsin inhibitor. Plasma (12 liters) was obtained by centrifugation of the blood at 4 ^{o}C for 25 min at 2500 rpm in a MSE Mistral-6L centrifuge. $BaSO_4$ (50 mg/ml) was added to the plasma and the suspension was stirred for 15 min at 4 ^{o}C. The plasma supernatant after $BaSO_4$ adsorption was adsorbed to QAE-Sephadex (1 liter of swollen gel equilibrated in 10 mM Tris, 100 mM NaCl, pH 7.5) for 30 min under gentle stirring. The QAE-Sephadex was filtered on a funnel overlaid with nylon cloth, washed with 10 liters of the equilibration buffer, resuspended in a buffer containing 10 mM Tris, 100 mM NaCl and 20% (v/v) glycerol, pH 7.5 and packed into a column (10 x 30 cm). Factor V was eluted with 10 mM Tris, 500 mM NaCl, 10 mM benzamidine, pH 7.5. The eluate was dialyzed against 10 mM Tris, 100 mM NaCl, 10 mM benzamidine, 20% (v/v) glycerol, pH 7.5 and applied to a DEAE-Sephadex column (5 x 30 cm) equilibrated with the dialysis buffer. The column was developed at 4 ^{o}C with a linear gradient from 100 mM NaCl in 20 mM Tris, 10 mM benzamidine, 20% (v/v) glycerol, pH 7.5 to 300 mM NaCl in 20 mM Tris, 100 mM benzamidine, pH 7.5 (3.0 liter/reservoir). Factor V activity eluted at 200-250 mM NaCl. The factor V containing fractions were diluted with an equal volume (normally 1 liter) of a buffer containing 20 mM potassium phosphate, 20 mM Tris, 150 mM sodium oxalate, 10 mM benzamidine, pH 7.0. Calcium chloride (2 M) was added slowly with stirring to a final concentration of 100 mM. This mixture was stirred for 30 min at 4 ^{o}C. The calcium oxalate and calcium phosphate precipitate was collected by centrifugation for 20 min at 2000 x g. Factor V activity was eluted with two times 250 ml of 200 mM potassium phosphate, 10 mM benzamidine, 2 mM DFP., pH 7.0. The eluate was saturated to 30% with ammonium sulfate, stirred for 30 min at 4 ^{o}C and then centrifuged for 20 min at 2000 x g. The factor V precipitated when the supernatant was saturated to 60% with ammonium sulfate. The precipitate was spun down and resuspended in a minimal volume of 20 mM Tris, 10 mM benzamidine, 1 mM DFP, pH 7.5 and applied to a column (5 x 100 cm) of Ultrogel AcA 22 equilibrated in 50 mM Tris, 400 mM NaCl, 10 mM benzamidine, pH 7.5. Factor V appeared after about 700 ml of eluate. The fractions of highest specific activity were pooled and stored at -80 ^{o}C.

Bovine factor Va (unfractionated factor Va) was obtained by incubating

0.3 mg/ml factor V with thrombin (0.2 μg/ml) in 50 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, pH 7.5 at 37 ^{o}C for 30 min. The activation of factor V was followed by assaying factor Va activity and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. After complete activation, the factor Va solution was diluted in 50 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, 0.5 mg/ml ovalbumin, pH 7.5, 1 : 50 and stored at -80 ^{o}C. The factor Va was stable for at least 6 months.

The factor Va subunits with molecular weights of 80,000 (factor Va LC) and 94,000 (factor Va HC) were isolated by a modification of the method of Esmon (1979). Factor Va (30 mg) in 20 mM Tris, 50 mM NaCl, 2 mM EDTA, pH 7.5 was applied to a SP-Sephadex column (1.5 x 30 cm) coupled to a QAE-Sephadex column (1.5 x 30 cm) both equilibrated with 20 mM Tris, 50 mM NaCl, 2 mM EDTA, pH 7.5. Subsequently, the SP-Sephadex and QAE-Sephadex columns were developed separately with linear gradients from 50 mM NaCl to 200 mM NaCl and from 50 mM NaCl to 500 mM NaCl (150 ml/reservoir), respectively. The factor Va LC was eluted from the SP-Sephadex (100 mM NaCl) and the factor Va HC was eluted from the QAE-Sephadex (350 mM NaCl).

Phospholipid Vesicle Preparations

Phospholipid vesicles were made from a mixture of 1,2-Dioleoyl-sn-glycero-3-phosphoserine and 1,2-Dioleoyl-sn-glycero-3-phosphocholine in a buffer containing 50 mM Tris, 100 mM NaCl, pH 7.5 as described earlier (Rosing et al., 1980).

Reconstitution of Factor Va from its Subunits (Factor Va LC and Factor Va HC)

Factor Va LC (0.3 mg/ml) in 50 mM Tris, 100 mM NaCl, 20 mM $CaCl_2$, 0.5 mg/ml ovalbumin, pH 7.5 and factor Va HC (0.4 mg/ml) in the same buffer were incubated at 37 ^{o}C. The time course of factor Va activity was followed by a factor V assay.

Factor V Assay

Factor V was assayed in a one-stage assay utilizing purified bovine coagulation factors. A sample (50 μl) was incubated with 50 μl of factor X_a

(0.1 nM), 50 μl of a mixture containing phospholipid (100 μM) and human fibrinogen (4 mg/ml) and 50 μl of 25 mM $CaCl_2$ for 30 s at 37 °C. The assay was initiated by the addition of 50 μl of prothrombin (5 μM). The time required for clot formation was measured. Samples were diluted in 50 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, pH 7.5, containing ovalbumin (0.5 mg/ml). The purified coagulation factors were in the same buffer. Pooled bovine oxalated plasma was used for the construction of standard curves. The assay had a functional range from 0.05 to 10 milliunits/ml (clotting times from 300 s to 60 s). One unit of factor V was defined as the amount present in 1 ml of bovine plasma.

Protein Concentrations

Factor Xa concentrations were determined by active site titration with p-NPGB according to Smith (1973). The molar concentrations of the other proteins were calculated from the following molecular weights and $E^{1\%}_{280\ nm}$: prothrombin 72,000, 15.5 (Owen et al., 1974); prethrombin 1, 50,200, 19.2 (Owen et al., 1974) factor V, 330,000, 9.6 (Nesheim et al., 1979a); factor Va LC, 80,000, 15.8 and factor Va HC, 94,000, 9.6 (Esmon, 1979).

Measurement of Rates of Prothrombin Activation

To determine the K_d and the stoichiometry of complex formation between factor Xa and factor Va we used a method in which initial rates of prothrombin activation are a measure for the amount of factor Xa.Va complex present in a reaction mixture (for a description of this method see below). Rates of prothrombin activation were calculated from the amounts of thrombin determined after different time intervals with the synthetic chromogenic substrate S-2238 as described previously (Rosing et al., 1980). The conversion of S-2238 by thrombin was followed by measuring the absorbance change on an Aminco DW-2 spectrophotometer operating in the dual-wave length mode ($\lambda_{mono\ 1}$ = 405 nm and $\lambda_{mono\ 2}$ = 500 nm).

Method to Determine the Binding Interaction between Factor Xa and Factor Va from Kinetic Measurements

Kinetic studies on prothrombin activation (Nesheim et al.,1979b) show that in the presence of factor Va, prothrombin activation is catalysed by an equimolar complex between factor Xa and factor Va. The initial assumption in this study is that the formation of the complex is described by equation 1.

$$Xa + Va \rightleftharpoons XaVa \quad (1)$$

The dissociation constant of the factor Xa-Va complex is

$$K_d = [(Va)_{free}(Xa)_{free}]/(XaVa) \quad (2)$$

When a fixed amount of factor Xa is titrated with factor Va

$$(Xa)_{free} = (Xa)_o - (XaVa) \quad (3)$$

in which $(Xa)_o$ is the total concentration of factor Xa present. Substituting equation 3 into equation 2 gives

$$K_d = (Va)_{free}[(Xa)_o - (XaVa)]/(XaVa) \quad (4)$$

which can be rearranged into

$$1/(XaVa) = [1/(Va)_{free}][K_d/(Xa)_o] + 1/(Xa)_o \quad (5)$$

When prothrombin is added to a reaction mixture in which factor Xa, factor Va and the factor Xa.Va complex are in equilibrium, conditions can be chosen such that the rate of prothrombin activation is proportional to the amount of factor Xa.Va complex present, since non-complexed factor Xa catalyses prothrombin activation at a rate negligable to that of the factor Xa.Va complex (Rosing et al., 1980). In that case the rate of prothrombin activation

$$v = c(XaVa) \quad (6)$$

in which c is a constant determined by the concentration of prothrombin present and the kinetic parameters of prothrombin activation by the factor

Xa.Va complex. When all factor Xa present is complexed with factor Va, (XaVa) = $(Xa)_0$ and the maximal rate of prothrombin activation is equal to

$$v_{max} = c(Xa)_o \tag{7}$$

Substituting equations 6 and 7 into equation 5 gives

$$1/v = [1/(Va)_{free}][K_d/v_{max}] + 1/v_{max} \tag{8}$$

When $(Xa)_o \ll K_d$, the amount of factor Va required for saturating factor Xa is in large excess over $(Xa)_o$ and $(Va)_{free} \approx (Va)_0$, where $(Va)_o$ is the total concentration of factor Va present. So,

$$1/v = [1/(Va)_o][K_d/v_{max}] + 1/v_{max} \tag{9}$$

An equation similar to equation (9) can be derived when a fixed amount of factor Va is titrated with factor Xa:

$$1/v = [1/(Xa)_o][K_d/v_{max}] + 1/v_{max} \tag{10}$$

A plot of 1/v versus either $1/(Va)_o$ or $1/(Xa)_o$ is linear with an intercept at the ordinate equal to $1/v_{max}$ and at the abcissa equal to $-1/K_d$. K_d and v_{max} were determined by statistical analysis of the data as described by Eisenthal and Cornish-Bowden (1974).

RESULTS

Comments on the Purification of Factor V and the Isolation of the End Products of the Thrombin-catalyzed Activation of Factor V

Factor V prepared by a modification of the method of Esmon (1979) as described in Materials and Methods is a single-chain polypeptide with an apparent M_r=350.000 as determined with SDS-gel electrophoresis (Figure 1). In contrast to Esmon (1979) we did not observe a tightly spaced doublet. The absence of the doublet in our preparations is due to the different way in which we obtained the blood as starting material (i.e.: venepunction of cows

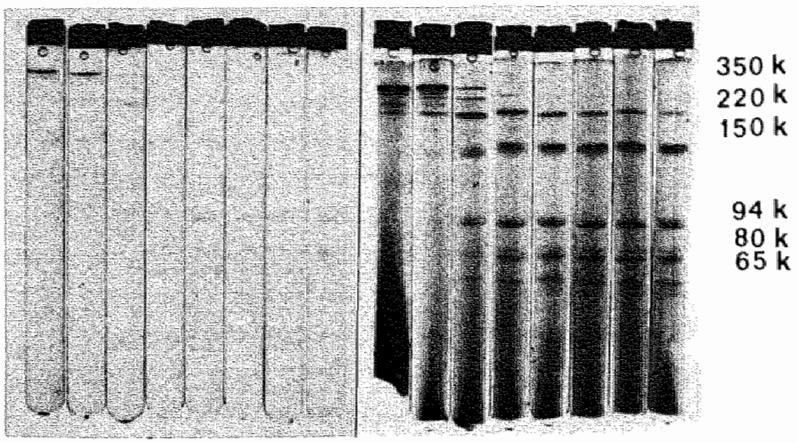
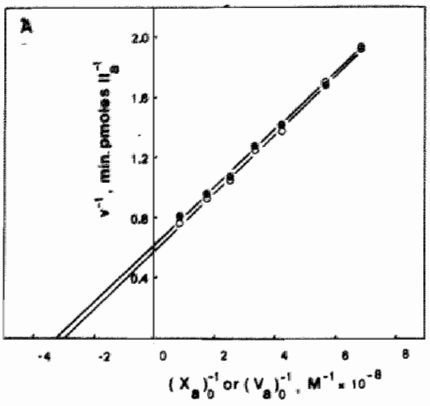
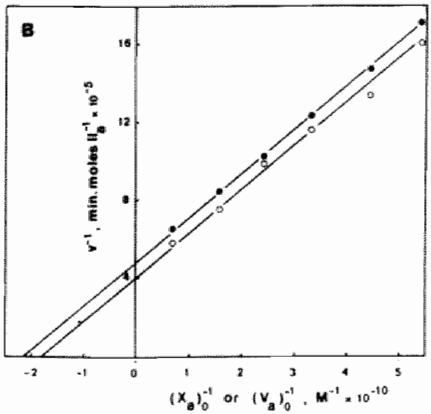


FIGURE 1: The activation time course of factor V by thrombin. Factor V (0.5 mg/ml) was incubated with thrombin (0.1 μg/ml) at 37 °C in 0.05 M Tris, 0.1 M NaCl, pH 7.5. Samples were removed from the reaction mixture at the times given below the gels and assayed for factor V activity. At the same time intervals aliquots (50 μl) of the incubation mixture were added to 50 μl of 2% sodium dodecyl sulfate, 10%(v/v) mercaptoethanol, 50 mM EDTA, pH 7.8 and kept for 2 min at 100 °C. Samples were electrophoresed on 7.5% SDS-acrylamide gels and stained with Coomassie blue (panel A) and with periodic acid Schiff's reagent (panel B).

treated with phenprocoumen versus slaughterhouse blood). We have purified factor V approximately 2000-fold (specific activity of 24 units per ml per absorbance unit at 280 nm) with a 20% yield.

The factor V preparation appears to be free of phospholipid contamination. Phospholipid as estimated by organic phosphate measurement according to the method of Böttcher et al. (1961) was undetectable in a sample containing 2 mg of factor V (less than 0.5 mole phospholipid per mole of factor V). In addition, incubation of factor V_a (1 μmole/l) with phospholipase A_2 from Naja naja (2 IU/ml) in the presence of 10 mM $CaCl_2$ for 30 min at 37 °C did not result in a change in factor Va activity as measured in the absence of phospholipid under the conditions as described in the legend to Figure 2A.





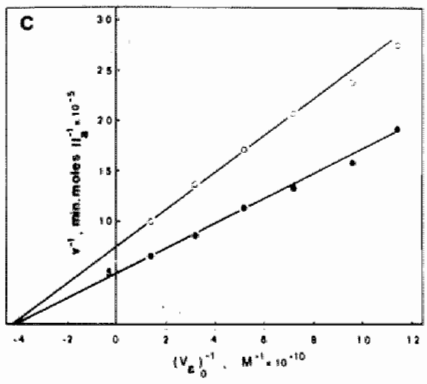


FIGURE 2: Double reciprocal plots of binding interaction of factor Va with factor Xa. Factor Xa and factor Va were incubated for 10 min at 37 °C either in the absence of phospholipid and 3 mM $CaCl_2$ or in the presence of phospholipid and 10 mM $CaCl_2$ in 1.0 ml of a buffer containing 50 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), pH 7.5. The prothrombin activation was started by the addition of 10 µl of prothrombin to give final concentrations as given below. After 2 and 4 min samples were taken and assayed for thrombin as described in Materials and Methods. Each point in the double reciprocal plot is the average of two independent measurements.

A gives the data for mixtures consisting of: either 3.5 x 10^{-10} M of factor Xa and 1.2 to 12.0 x 10^{-9} M of factor Va (○) or 3.5 x 10^{-10} M of factor Va and 1.2 to 12.0 x 10^{-9} M of factor Xa (●). Final prothrombin concentration is 0.2 µM. B gives the data for mixtures consisting of: either 2.1 x 10^{-12} M of factor Xa, 0.15 to 1.5 x 10^{-10} M of factor Va, 20 µM phospholipid (40% DOPS/60% DOPC), 10 mM $CaCl_2$ (○) or 2.0 x 10^{-12} M of factor Va, 0.15 to 1.5 x 10^{-10} M of factor Xa, 20 µM phospholipid (40% DOPS/60% DOPC), 10 mM $CaCl_2$ (●). Final concentration of prothrombin is 1.0 µM. C gives the data for mixtures consisting of: 2.1 x 10^{-12} M of factor Xa, 0.7 to 7.0 x 10^{-11} M of factor Va, 5 µM phospholipid (40% DOPS/60% DOPC), 10 mM $CaCl_2$ and prothrombin concentrations of 0.05 µM (○) and 1.0 µM (●).

When incubated with thrombin, our factor V preparations are activated 75-100 fold. The lower increase in specific activity reported by Esmon (1979), 10-16 fold, can be accounted for by the different factor V assays used. Analysis of the fully activated factor V by SDS-gel electrophoresis and staining of the gels with periodic acid-Schiff's reagent (PAS) and Coomassie blue reveals that activation of factor V by thrombin gives rise to four polypeptides with M_r=150,000; M_r=94,000; M_r=80,000 and M_r=65,000 (Figure 1). The polypeptides with M_r=150,000 and M_r=65,000 could only be detected by PAS-staining. The four polypeptides were isolated by ion-exchange chromatography as described in Materials and Methods. The polypeptide with M_r=80,000 eluted from the SP-Sephadex column (0.10 M NaCl). The polypeptides with Mr=65,000, Mr=94,000 and Mr=150,000 eluted from the QAE-Sephadex at 0.15 M, 0.35 M and 0.40 M NaCl, respectively. No factor Va activity could be detected in any of the column fractions. As reported by Esmon (1979), factor Va activity could be restored by mixing the SP-Sephadex fraction that contains the polypeptide with M_r=80,000 (Va LC) with the QAE-Sephadex fraction containing the polypeptide with M_r=94,000 (Va HC) in the presence of 20 mM $CaCl_2$. Therefore, we consider the polypeptides with M_r=65,000 and M_r=150,000 as activation peptides.

Binding Interaction between Factor Xa and Factor Va in Solution Inferred from Kinetics of Prothrombin Activation

The dissociation constant (K_d) of the factor Xa.Va complex was determined from kinetics of prothrombin activation as described in Materials and Methods. Initial rates of prothrombin activation were measured when a fixed amount of factor Xa is titrated with factor Va and vice versa. True initial rates were measured since lineairity of prothrombin activation was obtained at all factor Xa and factor Va concentrations used and the maximal amount of prothrombin converted never exceeded 2% of the prothrombin added. Factor Xa and factor Va were incubated for 10 min at 37 ^{o}C in the presence of Ca^{2+} ions prior to addition of prothrombin, since at the lowest factor Xa and factor Va concentrations employed in our experiments, complex formation was reached in 5 min.

The results of the titration experiments are shown in Figure 2A as a plot of the reciprocal value of the initial rate of prothrombin activation versus

the reciprocal concentration of the varied component (factor Va or factor Xa)*. The respective dissociation constants and maximal rates of thrombin formation are: K_d = 3.5 x 10^{-9} M, v_{max} = 5.4 min^{-1} for a fixed amount of factor Xa, and K_d = 3.1 x 10^{-9} M, v_{max} = 5.1 min^{-1} for a fixed amount of factor Va. Since in both titrations the amount of varied component was always in large excess over the amount of fixed component, a negligable fraction of the ligand is present in the complex which jusitifies calculation of the dissociation constants from the concentration of added ligand. The same K_d and v_{max} values obtained in titrations of factor Xa with factor Va and titrations of factor Va with factor Xa show that in solution an equimolar complex is formed between factor Xa and factor Va.

The Effect of Ca^{2+} on the Interaction between Factor Xa and Factor Va in Solution

As shown by Rosing et al. (1980), the rate of prothrombin activation by factor Xa in the presence of factor Va increases to an optimum when the Ca^{2+} concentration is increased from 0 to 3 mM. At higher Ca^{2+} concentrations the rate of prothrombin activation decreases again. The data presented in Figure 3 confirm these findings. In this figure the maximal rate of prothrombin activation is plotted as a function of the Ca^{2+} concentration. Maximal rates of prothrombin activation were calculated from double reciprocal plots obtained by titrating factor Xa with factor Va in the presence of varying amounts of Ca^{2+} ions. The same Ca^{2+} dependence was found when prethrombin 1 or decarboxyprothrombin were used instead of prothrombin (data not shown). Figure 3 also shows that the dissociation constant of the factor Xa.Va complex is independent of the Ca^{2+} concentration between 0.5 and 30 mM. Since at Ca^{2+} concentrations below 0.5 mM, rates of prothrombin activation were too low to determine a K_d, it could not be established whether there is an absolute Ca^{2+} requirement for the interaction of factor Xa with factor Va.

* When a fixed amount of factor Va was titrated with factor Xa a correction was made for the controbution of non-complexed factor Xa to the rate of thrombin formation. At the factor Xa concentration required to saturate 50% of the factor Va present the contribution of non-complexed factor Xa to the rate of prothrombin activation was 6% of the total activity measured.

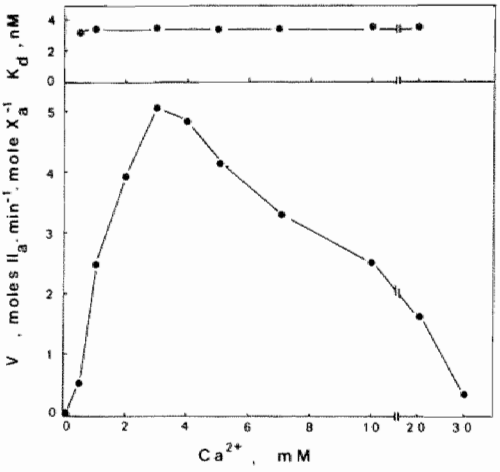


FIGURE 3: Effect of Ca^{2+} concentration on the v_{max} of thrombin formation and the dissociation constant of the factor Xa.Va complex. Factor Xa (3.5×10^{-10} M) was titrated with factor Va in the presence of varying amounts of Ca^{2+} as described in the legend to Figure 2. The ionic strenght of the reaction mixtures were kept constant by varying the NaCl concentration. The maximal rates of prothrombin activation (v, vertical axis lower panel) and the dissociation constant (K_d, vertical axis upper panel) were determined from double reciprocal plots of rate of thrombin formation versus added factor Va.

The Effect of Prothrombin on the Interaction between Factor Xa and Factor Va in Solution

Prothrombin interacts with factor Va, presumably through the fragment 2 region of the prothrombin molecule (Esmon et al., 1974). To examine whether an interaction between prothrombin and factor Va affects the formation of the factor Xa.Va complex in solution, we performed titrations of factor Xa with factor Va at various prothrombin concentrations. Factor Xa (0.32 nM) was titrated with factor Va at prothrombin concentrations between 0.09 and 3.0 μM. The prothrombin concentrations are (far) below the K_m for prothrombin (Rosing et al., 1980). The fact that the double reciprocal plots of rates of prothrombin activation versus total factor Va added are linear at all prothrombin concentrations used, indicates that initial rates of prothrombin activation are also under these conditions a measure for the amount of factor Xa.Va complex present.

Table I: The Influence of Prothrombin and Prethrombin 1 on the K_d of the Factor Xa.Va Complex

Substrate	conc (μM)	K_d[a] (nM)
Prothrombin	0.09	2.5
	0.20	3.7
	0.40	3.5
	1.40	4.9
	2.00	3.8
	3.00	4.6
Prethrombin 1	0.20	5.0
	1.00	4.6

[a] The dissociation constants were calculated from double reciprocal plots obtained by titrating factor Xa with factor Va as described in the legend to Figure 2A.

The K_d values for the factor Xa.Va complex calculated from these plots were independent of the amount of prothrombin present (Table I). From these results we conclude that the binding interaction between factor Xa and factor Va as inferred from kinetics is not affected by an interaction of prothrombin with factor Va and/or factor Xa.

The Effect of 40% DOPS - 60% DOPC Phospholipid Vesicles on the Interaction between Factor Xa and Factor Va

The binding data obtained in the absence of phospholipid show that factor Xa and factor Va form an equimolar complex with a dissociation constant of 3.3×10^{-9} M. Since both factor Xa and factor Va bind with a high affinity to negatively charged phospholipid vesicles (Nelsestuen et al., 1977; Bloom et al., 1979) it is likely that the presence of phospholipid affects in some manner the dissociation constant of the factor Xa.Va complex. From the fact

that much lower amounts of factor Va were required to optimize prothrombin activation in the presence of phospholipid than in solution, Rosing et al. (1980) suggested that phospholipid promote the formation of the factor Xa.Va complex. Also the kinetic studies of Nesheim et al. (1979b, 1981) and the binding studies of Tracy et al. (1981) indicate that factor Xa has an increased affinity towards phospholipid-bound factor Va.

To appreciate the assembling function of phospholipid in the formation of the factor Xa.Va complex, apparent dissociation constants at different phospholipid concentrations were determined by the kinetic method as described in Materials and Methods. Figure 2B shows the result of a typical experiment carried out in the presence of 20 μ M phospholipid (40% DOPS - 60% DOPC). Titrations were carried out both with factor Xa and factor Va as limiting component. The respective apparent dissociation constants and maximal rates of prothrombin activation are: $K_d = 5.5 \times 10^{-11}$ M, $v_{max} = 1230$ min^{-1} for a fixed amount of factor Xa and $K_d = 4.6 \times 10^{-11}$ M, $v_{max} = 1050$ min^{-1} for a fixed amount of factor Va*. Also in these experiments, the amount of ligand added, was always in large excess over the amount of limiting component. Therefore, it was justified to express the apparent K_d in terms of total added ligand. Under the same conditions but in the absence of phospholipid, the rate of thrombin formation is negligable as compared to the rate of thrombin formation in the presence of phospholipid. Therefore, the prothrombin activation measured is restricted to reactions taking place at the phospholipid surface and the result of interactions between factor Xa and factor Va at that surface. The data obtained in Figure 2B shows that a phospholipid surface promotes the formation of the factor Xa.Va complex by decreasing the dissociation constant about 100-fold.

* When a fixed amount of factor Va was titrated with factor Xa a correction was made for the contribution of non-complexed factor Xa to the rate of thrombin formation. At the factor Xa concentration required to saturate 50% of the factor Va present, the contribution of non-complexed factor Xa to the rate of prothrombin activation was 8% of the total activity measured.

Table II: Effect of Phospholipid Vesicles (40% DOPS - 60% DOPC) on the Apparent Dissociation Constant of the Factor Xa.Va Complex

Phospholipid conc (μM)	Prothrombin conc (μM)	(app)K_d [a] (M)	v_{max} [b]
2	1.0	1.39×10^{-11}	1190
5	1.0	2.30×10^{-11}	1000
10	1.0	2.84×10^{-11}	1280
25	1.0	4.51×10^{-11}	1600
50	1.0	9.29×10^{-11}	1380
100	1.0	15.80×10^{-11}	1100
125	2.0	18.90×10^{-11}	1590
150	3.0	27.00×10^{-11}	1580
300	6.0	52.20×10^{-11}	1780

[a] The apparent dissociation constants and v_{max} were calculated from double reciprocal plots obtained by titrating factor Xa with factor Va as described in the legend to Figure 2B.

[b] The rates are expressed in moles IIa.min^{-1}.mol Xa^{-1}

Binding parameters obtained from double reciprocal plots of titrations of a fixed amount of factor Xa with factor Va in the presence of varying amounts of phospholipid are listed in Table II. The apparent dissociation constant increases when the phospholipid concentration is raised. At phospholipid concentrations higher than 25 μM the increase of the dissociation constant is linear with the amount of phospholipid present.

Since the K_m for prothrombin varies with the phospholipid concentration (Rosing et al., 1980), the experiments presented in Table II were performed at variable amounts of prothrombin. The prothrombin concentrations employed were at least 4 times higher than the apparent K_m for prothrombin in order to approach the true V_{max} of prothrombin activation as close as possible.

As shown in Table II, the v_{max} values at different phospholipid concentrations are about equal. From this and the assumption that all factor Xa is saturated with substrate, we conclude that all factor Xa present in the reaction mixtures participates in a membrane-bound factor Xa.Va complex.
It can be argued that prothrombin might interfere with the formation of the factor Xa.Va complex at the phospholipid surface either by interaction with factor Xa and/or factor Va or by competition with factor Xa and/or factor Va for binding sites at the phospholipid surface. Since a 20-fold variation of the prothrombin concentration did not affect the apparent dissociation constant (Figure 2C), we conclude that prothrombin does not interfere with the assembly of the factor Xa.Va complex at the phospholipid surface. The increase of the v_{max} from 670 to 1000 moles thrombin.min^{-1}.mol factor Xa^{-1} presumably reflects substrate saturation of prothrombin activation, since the apparent K_m for prothrombin in the presence of 5 μM phospholipid is 0.06 μM. A phospholipid concentration of 5 μM was chosen for this experiment since it provides a limited number of binding sites for factor Xa and factor Va, which would have made competition, if occuring, better detectable.

The Interaction between Factor Xa and Factor Va at Phospholipid Vesicles with Varying Phosphatidylserine Content

It has been demonstrated that binding of both factor Xa and factor Va to membranes requires the presence of negatively charged phospholipids (Bloom et al., 1979; Nelsestuen et al., 1977). Nelsestuen et al. (1977) found that below ~20% phosphatidylserine the factor Xa binding capacity was directly proportional to the phosphatidylserine content. The data of Bloom et al. (1979) suggest that below 25% phosphatidylserine the binding capacity of factor Va is also directly proportional to the phosphatidylserine content. The characteristics of the formation of the factor Xa.Va complex at membranes containing varying amounts of phosphatidylserine are shown in Table III. The results show that the Kd (apparent) of the factor Xa.Va complex decreases up to about 20% phosphatidylserine and is then constant to 50% phosphatidylserine. The maximal rate of prothrombin activation did not change with the phosphatidylserine content.

Table III: Dissociation Constant Determined by Kinetics for the Interaction of Factor Xa with Factor Va in the Presence of Phospholipid Vesicles[a] of Varying Phosphohatidyl(PS) Content

Fraction PS (%)	Total lipid conc (μM)	K_d[b] (M)	Prothrombin conc (μM)	v_{max}[b]
5	10	33.0×10^{-11}	0.2	1040
	20	25.0×10^{-11}	0.5	950
	50	28.0×10^{-11}	1.0	1200
	100	28.0×10^{-11}	2.0	1200
	150	28.0×10^{-11}	2.0	1200
10	10	8.0×10^{-11}	0.2	1300
15	10	3.8×10^{-11}	0.2	1440
20	10	2.9×10^{-11}	0.2	1490
30	10	3.0×10^{-11}	0.2	1400
40	10	3.0×10^{-11}	0.2	1350

[a] Phospholipid vesicles were made as described in Materials and Methods

[b] The K_d and v_{max} were calculated from double reciprocal plots obtained by titrating factor Xa (2.0×10^{-12} M) with factor Va as described in the legend to Figure 2B.

Table II also shows that at high phosphatidylserine content (40%) the Kd of the factor Xa.Va complex increases proportional to the phospholipid concentration. At low phosphatidylserine content (5%) the Kd (apparent) is independent on the phospholipid concentrations, ranging from 50 to 150 μM. It is apparent that almost all factor Xa added binds to the phospholipid surface and can be saturated with factor Va, since maximal rates of thrombin formation were found to be independent of the phospholipid concentration and slightly less than those observed for vesicles with a high phosphatidylserine content.

Studies on the Interaction of Factor Va Subunits with Factor Xa.

In our studies we have used an unfractionated factor Va preparation which contains the biologically active factor Va, composed of two polypeptide chains with M_r=80,000 (Va LC) and M_r=94,000 (Va HC) and activation peptides with M_r = 150,000 and M_r=65,000. It is of interest to investigate whether the presence of those activation peptides affects the interaction between factor Va and

factor Xa and to examine whether restoration of biological activity correlates with the Ca^{2+}-mediated complex formation between Va LC and Va HC, showing the same binding characteristics towards factor Xa as does the "native" factor Va. Titration of factor Xa (0.5×10^{-9} M) with restored factor Va (1.0 to 10×10^{-9} M) was performed as described in the legend to Figure 2A. The K_d and v_{max} values calculated from a double reciprocal plot of the rate of prothrombin activation versus added Va HC.Va LC complex are presented in Table IV. A comparison between entries 1 and 2 indicates: a) the activation peptides have no effect on the interaction between factor Xa and factor Va and b) the binding characteristics of restored factor Va are the same as those of "native" factor Va. These findings strongly support the observations made by Esmon (1979) that two non-identical peptides derived from the pro-cofactor are required for biological function. These observations also suggest that the two-chain structure of factor Va is required for binding factor Xa and/or prothrombin.

Whether the interaction between factor Xa and one of the peptides is impaired because of the absence of the other peptide can easily be demonstrated. If one of the peptides forms a complex with factor Xa, this peptide is likely to be inhibitory, since it will competitively removes factor Xa from interaction with factor Va, thereby reducing the concentration of the functional enzyme (factor Xa.Va complex) in prothrombin activation. Table IV, entries 3 and 4, clearly demonstrates that in the presence of a large molar excess of Va LC or Va HC over factor Va, the dissociation constants and v_{max} values are similar to those found in the absence of each of the chains. It is apparent that neither Va HC nor Va LC are able to compete with factor Va in a complex formation with factor Xa. From these date we conclude that factor Xa binding to factor Va requires a Ca^{2+} mediated interaction between the polypeptides with Mr=80,000 and Mr=94,000 of which factor Va is composed.

Table IV: Effect of Factor V Activation Fragments on the Binding Interaction between Factor Va and Factor Xa[a]

	factor Xa (nM)	factor Va (nM)	Fragments	conc (nM)	K_d (nM)	v_{max}[b]
1.	0.35	1.0-10.0	none		4.0	4.7
2.	0.35	none	Restored factor Va	1.0-10.0	3.5	4.3
3.	0.35	1.0-10.0	Va LC	50	3.7	5.0
4.	0.35	1.0-10.0	Va HC	100	3.3	4.5

[a] K_d and v_{max} values were determined from double reciprocal plots of rates of thrombin formation versus added factor Va or restored factor Va. Further experimental details are described in the legend to Figure 2A.

[b] Rates expressed as moles IIa.min^{-1}.mol Xa^{-1}.

DISCUSSION

The interaction between factor Xa and factor Va in the presence of phosphatidylserine-phosphatidylcholine vesicles and platelets has been studied in several laboratories (Nesheim et al., 1979b; Kane et al., 1980; Tracy et al., 1981). The results obtained suggest a coordinate binding of factor Xa and factor Va to phospholipid and platelets and the formation of a stoichiometric (1:1) complex. The dissociation constant for factor Xa dissociation from the phospholipid-bound complex (K_d $\sim 10^{-10}$ M) is several orders of magnitude less than the dissociation constant for factor Xa-phospholipid interaction (K_d $\sim 10^{-6}$ M) (Nelsestuen et al., 1977; Bloom et al., 1979). Based on these obser

vations it has been postulated that binding of factor Va to phospholipid increases the binding affinity for factor Xa. Therefore, relatively high concentrations of factor Xa are obtained in the vicinity of phospholipid vesicles and platelets when factor Va is present (Nesheim et al., 1981).

There exists, however, no insight in the interactions responsible for the increased affinity of factor Xa for phospholipid-bound factor Va. The question is, to what extent factor Xa-phospholipid, factor Va-phospholipid and protein-protein interactions contribute to the increased affinity of phospholipid-bound factor Va for factor Xa.

The studies presented in this paper were initiated in order to disclose the contribution of both protein-protein and protein-phospholipid interactions to the high-affinity of the phospholipid-bound factor Va for factor Xa.

The method used to study the interaction between factor Xa and factor Va is based on the assumption that a factor Xa.Va complex is the functional enzyme in prothrombin activation (Nesheim et al., 1979b; Rosing et al., 1980) and that the rates of prothrombin activation by factor Xa in the presence of factor Va are proportional with the amount of factor Xa.Va complex in the reaction mixture. In the absence of phospholipid, titrations of a fixed amount of factor Xa with factor Va or vice versa yield at saturation the same maximal rates for prothrombin activation per mol of fixed component (Figure 2A). The same observation was made in titration experiments in the presence of phospholipid (Figure 2B). Since factor Va is a non-enzymatic component, these results indicate that both in the absence and presence of phospholipid, the catalytic unit in the prothrombin activation is a stoichiometric (1:1) complex of factor Xa and factor Va.

Because prothrombin has the ability to interact with factor Va through the fragment 2 region of the prothrombin molecule (Esmon et al., 1974) and factor Va amplifies the turnover of prothrombin by factor Xa by not yet disclosed means (Rosing et al., 1980), the question arises whether prothrombin interferes with the interaction between factor Xa and factor Va. Given the following observations it seems unlikely that prothrombin participates in the formation of the factor Xa.Va complex: 1) The dissociation constant of the factor Xa.Va complex is independent of the prothrombin concentration when the molar ratios of prothrombin to factor Va were varied from 9 to 3000 (Table I), 2) In the presence of phospholipid, the apparent dissociation constant was also independent on the prothrombin concentration (Figure 2C)

and 3) Nesheim et al.(1981) showed that the binding parameters of the interaction between factor Xa and phospholipid-bound factor Va inferred from alterations in the rate of hydrolysis of S2222 are similar to those obtained with prothrombin as a substrate.

Rosing et al. (1980) demonstrated that the generation of thrombin, upon incubation of prothrombin with factor Xa and factor Va, strongly depends on the Ca^{2+} concentration, with an optimal Ca^{2+} concentration around 3 mM. Our results suggest that Ca^{2+} ions are not participating in the factor Xa.Va complex formation but presumably affect the enzymatic properties of the complex, because the Kd of the complex is independent on the Ca^{2+} concentration. The same Ca^{2+} dependence was found when substrates, lacking γ-carboxyglutamic acid residues were used. This suggests a role of Ca^{2+} in the enzymatic properties of the complex appart from its interaction with the γ-carboxyglutamic acid residues of the substrate.

Phospholipid does decrease the dissociation constant of the factor Xa.Va complex. When a fixed amount of factor Xa is titrated with factor Va in the presence of 25 μM phospholipid (40% DOPS-60% DOPC) and 10 mM $CaCl_2$, the K_d is 5.7×10^{-11} M, which is about two orders of magnitude less than the K_d determined in the absence of phospholipid. A considerable increase of the K_d is measured when the amount of phospholipid is increased from 25 μM to 300 μM (Table II). The K_d increases almost linear with the phospholipid concentration.

We demonstrated that the Kd is also a function of the phosphatidylserine content of the membrane. The dependence of K_d on the phosphatidylserine content may reflect the phospholipid-binding affinities of factor Xa and/or factor Va, because binding affinities of both factor Xa and factor Va have been reported to decrease proportional with the phosphatidylserine content of the membrane below 20% phosphatidylserine (Nelsestuen et al., 1977; Bloom et al, 1979). It is interesting to note that the K_d becomes independent of the phospholipid concentration at a low phosphatidylserine content of the vesicle (Table III). Thus the Kd that describes the interaction between factor Xa and factor Va in the presence of phospholipid is an apparent K_d depending both on the phospholipid concentration and the composition (phosphatidylserine content) of the phospholipid vesicles.

The precise manner by which phospholipid promotes complex formation is not known. Two models have been postulated:

1. Phospholipid-binding increases the local concentrations of the reactants and as a result the equilibrium of complex formation shifts into the direction of association (Rosing et al., 1980).
2. The interaction between the reactants is direct in the three-dimensional sense. The increased affinity would result from additive effects of protein-phospholipid and protein-protein interactions (Nelsestuen, 1978).

Thus, the formation of the factor Xa.Va complex will depend on the factor Va concentration on the phospholipid surface (model 1) or on the concentration of factor Va in the bulk solution (model 2).

Given the published binding parameters (Bloom et al., 1979) and the amount of factor Va added required to achieve half-saturation of phospholipid-bound factor Xa (apparent K_d value), the concentration of phospholipid-bound factor Va and the concentration of factor Va in bulk solution can be calculated (Table V). The dependency of the apparent K_d of the phospholipid concentration and phosphatidylserine content of the vesicle can be rationalized quantitatively in the context of both models.

Model 1. The affinity of factor Va for vesicles increases up to about 20% phosphatidylserine. In order to obtain a factor Va concentration of 1 - 2 μmole factor Va per mole phospholipid at the phospholipid surface required to achieve half-saturation of phospholipid-bound factor Xa, less factor Va has to be added with increasing phosphatidylserine content. Increased affinity also implies that the phospholipid concentration might become inhibitory. The amount of added factor Va has to increase with the increase in phospholipid concentration to attain 1 - 2 μmole factor Va per mole phospholipid at the surface.

Model 2. Depending of the affinity of factor Va for the phospholipid surface, an increase in phospholipid concentration will competitively remove factor Va from interaction with the factor Xa-phospholipid site. Consequently, the amount of added factor Va has to increase with increase in phospholipid concentration to attain a factor Va concentration in the bulk solution to achieve half-saturation of phospholipid-bound factor Xa.

Table V: Apparent Dissociation Constant for the Interaction of Factor Xa with Factor Va and Corresponding Factor Va Concentration on the Phospholipid Surface and Factor Va Concentration in Solution at Varying Phospholipid Concentrations and Phosphatidylserine Content of DOPC-DOPS Vesicle

Phospholipid	conc (μM)	Kd(app) ($MX10^{11}$)	$(Va)_{free}$ ($MX10^{11}$)	Factor Va concentration on phospholipid surface (μmoles/mole)
40% DOPS-60% DOPC	2	1.4	0.99	2.0
	5	2.3	1.15	2.3
	10	2.9	0.95	1.9
	25	4.5	0.75	1.5
	50	9.3	0.85	1.7
	100	15.8	0.75	1.5
	125	18.9	0.73	1.5
	150	27.0	0.87	1.7
	300	52.2	0.86	1.7
5% DOPS-95% DOPC	10	33.0	31.7	1.3
	20	25.0	18.5	0.8
	50	28.0	23.3	1.0
	100	28.0	20.0	0.8
	150	28.0	17.5	0.7

The combined results of this study support the notion that the enzymatic component of the prothrombin converting complex consists of an equimolar phospholipid-bound complex of factor Xa and factor Va. In addition, the "quality" of the phospholipid surface (phosphatidylserine content) upon which the factor Xa.Va complex is formed can act as a regulator of the catalytic activity of the prothrombinase complex. Therefore, our data have to be taken into consideration in a discussion about the physiological significance of

negatively charged phospholipids exposed on the outer surface of thrombin/collagen activated platelets (Bevers et al., 1982).

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CHAPTER III

INTERACTION OF BOVINE BLOOD CLOTTING FACTOR Va AND ITS SUBUNITS WITH PHOSPHOLIPID VESICLES

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Thrombin-activated factor Va and factor Va subunit binding to large-volume vesicles was investigated by a technique based on the separation by centrifugation of phospholipid-bound protein from the bulk solution. This technique allows the direct measurement of free protein concentration. It is concluded that the phospholipid-binding site on factor Va is located on a basic factor Va subunit with M_r=80,000 (factor Va LC). The effects of phospholipid vesicle composition, calcium concentration, pH and ionic strength on the equilibrium constants of factor Va- and factor Va LC phospholipid interaction were studied. Factor Va and factor Va LC binding to phospholipid requires the presence of negatively charged phospholipids. It is futher demonstrated that: a) Calcium ions compete with factor Va and factor Va LC for phospholipid-binding sites. b) The dissociation constant of protein-phospholipid interaction increases with the ionic strength, whereas the maximum protein-binding capacity of the phospholipid vesicle was not affected by ionic strenght. c) The dissociation constant for factor Va-phospholipid interaction depends on pH when the vesicle consists of phosphatidic acid. It is concluded that factor Va-phospholipid interaction is primarily electrostatic in nature, where positively charged groups on the protein directly interact with the phosphate group of net negatively charged phospholipids. The results suggest that factor Va, like factor Xa and prothrombin has the characteristics of an extrinsic membrane protein.

INTRODUCTION

The prothrombinase complex catalyzes the proteolytic conversion of the zymogen prothrombin to the serine protease thrombin. The components which constitute the complex are: factor Xa (the catalytic component), calcium ions, phospholipid and factor Va [see Jackson & Nemerson (1980) for a review]. Factor Va, which is derived by limited proteolysis of a single chain precursor of M_r=330,000, functions as a cofactor (Esmon, 1979; Nesheim et al., 1979b). The effects of factor Va and phospholipids on the kinetic parameters of prothrombin activation has given insight in the mode of action of nonenzymatic cofactors in prothrombin activation (Rosing et al., 1980).

Knowledge of the molecular details of the assembly of the prothrombinase complex is of importance in order to understand the mechanism of prothrombin activation at a phospholipid-water interface. From the studies reported by Nelsestuen and co-workers a detailed model of the prothrombin- and factor Xa-membrane complexes can be drawn (Lim et al., 1977; Resnick & Nelsestuen, 1980; Wei et al., 1982).

The nature of the factor Va-phospholipid interaction has still to be disclosed. In most previous studies, gelfiltration has been used to elucidate the factor V(a)-phospholipid binding characteristics. On the basis of these qualitative studies it appeared essential to have net negative charged phospholipids in the membrane for factor V-membrane interaction (Subbaiah et al., 1976). In addition, the possibility that the binding of factor V to membranes is mediated by calcium ions, can be ruled out (Greenquist & Colman, 1975; Subbaiah et al., 1976). In essence, those observations were confirmed in a study which utilizes factor V of high purity and a method of analysis that yields quantitative equilibrium binding data (Bloom et al., 1979). It was suggested that factor Va-membrane interaction is nonelectrostatic in nature. The role of phosphatidylserine in the protein-lipid nonelectrostatic interaction could not be explained (Bloom et al., 1979).

The purpose of this study is to establish the mode of action of acidic phospholipid in the factor Va-phospholipid interaction. We utilized large-volume unilamellar vesicles, which can be separated from the bulk solution by centrifugation for determination of binding parameters of the factor Va- and factor Va subunit-phospholipid interaction. The binding technique presented

here, is a valuable tool in the study of the effect of phospholipid composition, pH, ionic strength and calcium on factor Va-phospholipid interaction.

MATERIALS AND METHODS

S 2238 and S 2337 were purchased from AB Kabi Diagnostica. Soybean trypsin inhibitor (type I-S), Echis carinata venom, Russell's viper venom, ovalbumin and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained from Sigma. N-succinimidyl 3-(4-hydroxy, 5-[^{125}I] iodophenyl) propionate was from Amersham.

Preparation of Proteins

The following bovine blood coagulation factors were prepared: factor X_2 (Fujikawa et al., 1972a), factor Xa (Fujikawa et al., 1972b), prothrombin (Owen et al., 1974), thrombin (Rosing et al., 1980) and factor V and factor Va (Lindhout et al., 1982). RVV-V was isolated as described by Kisiel (1979). ^{125}I-factor V was prepared according to Bolton and Hunter (1973) using 10 mCi of ^{125}I per mg of protein. The specific radioactivity of ^{125}I-labeled factor V was 2610 cpm/ng (0.4 mole ^{125}I/ mole factor V). ^{125}I-factor V retained about 90% of its activity and had the same electrophoretic mobility on sodium dodecyl sulfate ($NaDodSO_4$)gel electrophoresis prior to and after labeling.

Protein concentrations

Determination of prothrombin by enzymatic assay was carried out after conversion to thrombin. Typically, 5 to 100 nM of prothrombin in 50 mM Tris, 100 mM NaCl, 0.5 mg ovalbumin/ml (pH 7.5) was incubated with Echis carinata venom (4 μg/ml) for 10 min at 37 ^{o}C. Within this time complete conversion of prothrombin into thrombin was obtained. The molar concentration of thrombin formed was measured as decribed by Rosing et al. (1980). The concentration of prothrombin as determined by the enzymatic assay agrees very well with that calculated from the $E_{280}^{1\%}$ using $E_{280}^{1\%}$ = 15.5. and 72,000 for the molecular weight of prothrombin (Owen et al., 1974).

The concentration of factor Xa was determined as described by van Dieijen et al. (1981).

Factor Va Assay

The factor Va assay is based on the formation of an equimolar factor Va - factor Xa complex as the catalytic unit in prothrombin activation (Lindhout et al., 1982). Under conditions where factor Xa is far in molar excess over factor Va, the rate of prothrombin activation is linear with the concentration of factor Va. A factor Va containing sample after the appropriate dilution in 50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, 0.5 mg ovalbumin/ml (pH 7.5) was incubated with factor Xa (1.3×10^{-11} M), phospholipid (20% DOPS/ 80% DOPC, 10 μM) and $CaCl_2$ (5 mM) in 50 mM Tris, 100 mM NaCl, 0.5 mg ovalbumin/ml (pH 7.5) for 5 min at 37 °C in a plastic cuvette. Prothrombin activation was started by the addition of 0.1 ml of prothrombin (2 μM). The final volume is 1.0 ml. After 2 min the reaction was stopped by the addition of 1.0 ml buffer containing 50 mM Tris, 100 mM NaCl, 0.5 mg ovalbumin/ml, 20 μg of STI, 0.47 μmol of S 2238 (pH 7.5). The amount of thrombin formed was measured by the change in absorbance recorded on an Aminco DW-2 spectrophotometer operating in the dual-wavelength mode (λ_s = 405 nm and λ_r = 500 nm) thermostated at 37 °C. Standard curves were constructed by assaying dilutions of a factor Va preparation of which the concentration was determined by titration with active-site titrated factor Xa (Lindhout et al., 1982). The assay had a functional range from 2×10^{-14} M to 1.0×10^{-12} M of factor Va. The functional factor Va concentration was about 95% of the concentration as determined from $E_{280}^{1\%}$ using $E_{280}^{1\%}$ = 9.6 and 330,000 for the molecular weight of unfractionated factor Va (Nesheim et al., 1979a).

The concentration of factor V was determined by the factor Va assay after activation with thrombin. Typically, the activation reached completion within 10 min at 37 °C using a factor V-thrombin molar ratio of 200:1.

The concentration of factor Va LC was estimated by its ability to restore factor Va activity when incubated with factor Va HC in the presence of calcium (Lindhout et al., 1982). Since neither one of the polypeptide chains of which factor Va is composed, exhibits factor Va activity, maximum factor Va activity, as a result of saturation of factor Va LC with factor Va HC, gives the molar concentration of factor Va LC preparations from a factor Va

standard curve. Knowing the specific radioactivity (cpm/μmol), the molar concentration of ^{125}I-factor Va LC can be estimated from radioactivity measurements.

Phospholipid and Phospholipid Vesicle Preparation

1,2-Dioleoyl-sn-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphatidic acid (DOPA) were prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as described by Comfurius & Zwaal (1977).

Sonicated vesicle solutions were prepared from mixtures of DOPS and DOPC in a buffer containing 50 mM Tris, 100 mM NaCl (pH 7.5) as described by de Kruijff et al. (1975) using a MSE Mark II ultrasonicator set at 7 μ amplitude.

Large-volume vesicles were prepared by a modification (Van der Steen et al., 1981) of the ether injection method according to Deamer & Bangham (1976). These large (300 nm diameter) vesicles are unilamellar (Van der Steen et al., 1981). In case the vesicles were used in ionic strength studies, the buffer contained the appropriate NaCl concentration. In order to remove residual ether, the vesicle solutions were dialyzed overnight at 4 ^{o}C with several changes of buffer. Large membrane structures, were removed by centrifugation for 10 min at 4000xg and 20 ^{o}C. The supernatant was centrifugated for 30 min at 30,000xg and 20 ^{o}C. The pellet was resuspended in 50 mM Tris, 100 mM NaCl (pH 7.5).

Thin-layer chromatography of the vesicle preparation as carried out according to Broekhuyzen (1969) showed that no alterations did occur in the membrane composition as a result of the procedure of vesicle preparation.

The internal volume of the vesicles was determined from the amount of trapped arsenazo III, according to Serham et al. (1981). For the trapped volume of vesicles containing 20% DOPS and 80% DOPC 10-14 l per mole lipid was found, which agrees with the value reported by Deamer & Bangham (1976).

Whenever a membrane composition is expressed as a percentage of acidic phospholipid, the remaining phospholipid is DOPC.

Procoagulant activity of phospholipid vesicles was determined under the same conditions as described for the factor Va assay. A phospholipid containing sample was incubated with factor Xa (1.3×10^{-11} M) and factor Va (5.0×10^{-10} M). Standard curves were constructed by assying dilutions of phospholipid of which the concentrations were determined by phosphate ana-

lysis (Böttcher et al., 1961). The assay had a functional range from 1 to 100 nM.

Protein Binding Measurements

Mixtures of large-volume vesicles and protein (0.2-0.5 ml) were incubated in 1.5-ml Eppendorf conical centrifuge tubes for 10 min at ambient temperature (20-23 ^{o}C). Prior to and after centrifugation for 30 min at 30,000xg and 20 ^{o}C, small aliquots (10 μ l) were withdrawn and assayed for protein as described in this section for determination of total protein concentration and the concentration of unbound protein, respectively. The phospholipid concentration in the supernatant was less then 0.1% of the total amount added, as determined by the phospholipid procoagulant activity assay. Care has to be taken to avoid non-specific binding and/or loss in activity during the experiment. Control experiments in the absence of phospholipid established that prior to and after centrifugation the recovery of protein was virtually 100%. Moreover, prothrombin and factor Xa binding by the large-volume vesicles were reversible as shown by the addition of EDTA to dissolve the complex after centrifugation, followed by resuspending the vesicles and a second spin down. It was also checked that phospholipid, carried over from the reaction mixture prior to centrifugation, had no effect on the protein assays.

Protein binding data were analysed by double-reciprocal plots using the following equation:

$$(1/B) = (1/F).(K_d/n) + (1/n)$$

in which B and F are concentrations of bound protein and free protein, respectively. The dissociation constant, K_d, and maximum protein-binding capacity of the membrane, n, were calculated from the horizontal and vertical intercept, respectively.

RESULTS

Prothrombin, Factor X, Factor Xa, Factor V and Factor Va Phospholipid Binding

Various amounts of protein (0.02 to 0.5 μM) were incubated with large-volume vesicles containing 20% DOPS, in 50 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, 0.5 mg ovalbumin/ml for 10 min at roomtemperature prior to centrifugation. From the measured equilibrium concentration of free protein, the binding parameters were calculated as described under Materials and Methods. The factor V binding experiments were carried out with ^{125}I-factor V. Different phospholipid concentrations were used to ascertain that the binding parameters are independent of the phospholipid concentration. The binding parameters obtained are listed in Table I, together with the values reported for prothrombin and factor X (Nelsestuen & Broderius, 1977) and factor V and factor Va (Bloom et al., 1979). The K_d values for prothrombin, factor X and factor Xa, agree very well with the K_d values determined from light scattering experiments (Nelsestuen & Broderius, 1977). In contrast to the data reported by Bloom et al. (1979), we found that factor Va binds to phospholipid with about 5 times the affinity of factor V. The reason for this difference is not known. Eight independent factor Va-phospholipid binding analysis, using different factor Va and vesicle preparations, gave a dissociation constant of $(5.6 \pm 1.1) \times 10^{-8}$ M (mean $\pm$ S.D.).

As shown in Table I, the maximum protein-binding capacity of membranes, n, did not vary with the phospholipid concentration. The n values, in particular for prothrombin-phospholipid interaction, are higher in this study than by light scattering (Bloom et al., 1979; Nelsestuen & Broderius, 1977). This may reflect some heterogeneity with respect to the unilamellar nature of the vesicles. Light scattering experiments show that the proteins used in this study does not induce vesicle-vesicle interaction. Therefore, an underestimation of the n values seems unlikely.

From these experimental results we conclude that the technique presented here is a valuable tool for the determination of equilibrium constants for protein-lipid interaction and that yields equilibrium constants that agree very well with those determined by relative light scattering experiments.

Table I: Phospholipid-Binding Properties of Prothrombin, Factor X, Factor Xa, Factor V and Factor Va

protein	total lipid[a] concn. (μM)	K_d ($Mx10^{-7}$)	n[b]
prothrombin	100	1.9	240
	50	2.0	255
	35[c]	6.0[e]	100[e]
factor X	50	1.9	91
	25	1.7	91
	35[c]	2.5[e]	80[e]
factor Xa	50	1.7	91
	25	2.0	80
factor V	100	2.8	416
	50	2.7	385
	33[d]	0.7[f]	270[f]
factor Va (thrombin activated)	50	0.44	84
	25	0.58	100
	33[d]	4.3[f]	76[f]
factor Va (RVV-V activated)	50	0.63	150

[a] 20% DOPS/80% DOPC. [b] phospholipid to protein ratio at saturation (moles/mole). [c] 20% PS (bovine brain)/80% PC (egg yolk)/1% cerebrosides. [d] 25% Folch fraction III/75% soybean PC. [e] Data taken from Nelsestuen & Broderius (1977). [f] Data taken from Bloom et al. (1979).

Identification of the Phospholipid-Binding Subunit of Factor Va

The fact that factor Va-membrane interaction depends on the presence of acidic phospholipids and factor Va consists of a cationic and anionic com-

ponent (Esmon, 1979) might indicate that factor Va has a positively charged area which directly interacts with net negatively charged phospholipid. In order to find evidence for this hypothesis, it was first necessary to separate the chains of factor Va. Whereas cleavage of the RVV-V-sensitive bond results in complete activation of factor V, activation of factor V by thrombin requires several bond cleavages and give rise to at least four different fragments (Lindhout et al., 1982; Kane et al., 1982). In order to avoid interference in our binding studies of contaminating radiolabeled peptides, which might be present in the isolated subunits derived from thrombin activated factor Va, with our binding experiments, we prefered to study the phospholipid-binding properties of the subunits of which RVV-V activated factor Va is composed. To this end, ^{125}I-factor V was incubated with RVV-V and after completion of the reaction, the activation fragments were separated by ion-exchange chromatography. Factor V upon incubation with RVV-V is converted into two polypeptide chains with M_r 270,000 and M_r 80,000. A 30-fold increase in factor Va activity was obtained. The peptides were separated by ion-exchange chromatography (Lindhout et al., 1982). The 80,000 dalton and 270,000 dalton peptide eluted at 50 mM NaCl and 250 mM NaCl, respectively. Neither peptide possessed factor Va activity after incubation for 2 h at 37 ^{o}C in the presence of 25 mM $CaCl_2$. The respective specific radioactivities are 5790 cpm/μg for ^{125}I-factor Va HC and 1280 cpm/μg for ^{125}I-factor Va LC.

The different charge properties of the peptides were studied by isoelectrofocusing on LKB Ampholine PAG plates (pH range 3.5-9.0). The isoelectric point of factor Va HC was about 5.5, whereas factor Va LC was detected at the extreme cathodic position, indicating that the isoelectric point of factor Va LC must be higher than 9.0.

The binding of ^{125}I-factor Va LC and ^{125}I-factor Va HC to vesicles containing 20% DOPS was measured as a function of the phospholipid concentration. Plotted in Figure 1 is the percentage of added protein bound versus the phospholipid concentration. At the highest phospholipid concentration virtually no factor Va HC was bound, whereas half of the amount of added factor Va LC was bound at 5 μM phospholipid. It can be questioned whether factor Va HC labeling on the amino groups does not destroy lipid binding. However, the factor Va activity that could be restored after incubation of the supernatants of the factor Va HC vesicle mixtures with factor Va LC in the presence of calcium was independent on the phospholipid concentration. This indicates

that no detectable amounts of factor Va HC was bound to phospholipid vesicles. These results ascertains that the the phospholipid binding-site of factor Va is located on a basic factor Va subunit with M_r 80,000.

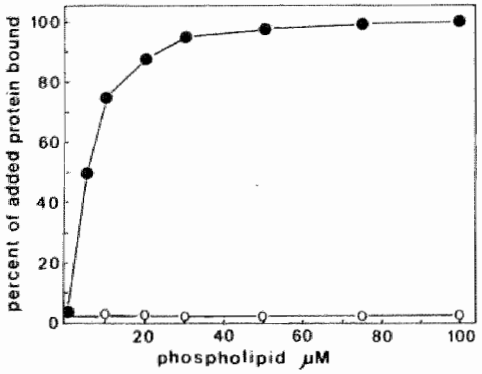

FIGURE 1: Binding of ^{125}I-factor Va HC and ^{125}I-factor Va LC as a function of phospholipid concentration. The phospholipid is 20% DOPS in 50 mM Tris, 100 mM NaCl, 1 mg ovalbumin/ml (pH 7.5). ^{125}I-factor Va HC [(O), 27 μg/ml] or ^{125}I-factor Va LC [(●), 8.2 μg/ml] was added and free and bound protein concentrations were estimated as described under Materials and Methods.

Factor Va LC Binding as a Function of Phosphatidylserine Content

The results of factor Va binding experiments with vesicles of varying Folch fraction III content reported by Bloom et al. (1979) suggest that the binding affinity of factor Va is directly proportional to Folch fraction III content in the region below ~25% Folch fraction III (or ~20% phosphatidylserine). To determine whether factor Va LC phospholipid interaction is also dependent on the presence of acidic phospholipid, we carried out binding experiments with large-volume vesicles of varying DOPS content. Various amounts of ^{125}I-factor Va LC (0.1 to 1.0 μM) were incubated with 20 μM phospholipid (DOPS content ranging from 0 to 30%) in 50 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 1 mg ovalbumin/ml (pH 7.5) for 10 min at room temperature prior to centrifugation. The equilibrium concentrations of free and bound ^{125}I-factor Va LC were determined as described under Materials and Methods. The binding parameters were calculated from double reciprocal plots of free factor Va LC vs. bound factor Va LC. Under the same conditions, we carried out factor Va binding experiments. The binding parameters obtained are presented in Table II.

Factor Va and factor Va LC do not bind to 100% DOPC vesicles. It is apparent that the binding affinity of both factor Va and factor Va LC increases with increase in DOPS content of the membrane. Moreover, the affinities of factor Va LC and factor Va are nearly identical. The maximum protein binding capacity also appears to increase with the increase of DOPS content of the membrane. Therefore, the factor limiting both factor Va and factor Va LC binding is assumed to be availability of phosphatidylserine. Packing of protein on the membrane at high phosphatidylserine content may be a limiting factor in factor Va and factor Va LC binding. However, our experiments do not allow further conclusions on this subject.

Table II: Factor Va and Factor Va LC Binding to Vesicles of Varying DOPS Content

DOPS	factor Va		factor Va LC	
%	K_d(μM)	n[a]	K_d(μM)	n[a]
0	no binding		no binding	
5	0.42	152	0.50	71
10	0.25	115	0.13	56
15	0.071	80	0.030	48
20	0.042	71	ND[b]	
30	0.020	75	0.018	40

[a] Phospholipid to protein ratio at saturation (moles/mole)

[b] ND, not determined

Effect of Calcium on Factor Va and Factor Va LC Phospholipid Interaction

In view of the phosphatidylserine dependent binding of the net positively charged factor Va subunit, it is of particular interest to examine the effect of calcium on quantitative equilibrium binding data. If, the interaction of factor Va with phospholipid is essential an ionic process, ionic shielding of

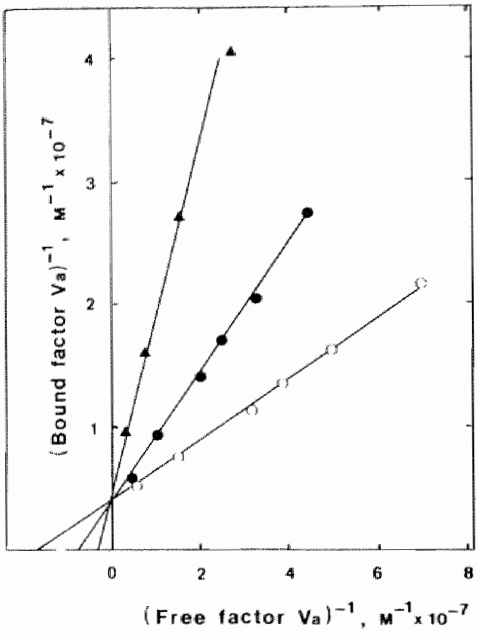

FIGURE 2: Double reciprocal plots of factor Va-phospholipid binding at varying calcium concentrations. Free and bound factor Va concentrations were estimated as described under Materials and Methods. Large-volume vesicles of 20% DOPS (20 μM) in 50 mM Tris, 100 mM NaCl, 1 mg ovalbumin/ml, pH 7.5 were used. The plots show data obtained at the following calcium concentrations: (○) 0.5 mM $CaCl_2$; (●) 5 mM $CaCl_2$; (▲) 10 mM $CaCl_2$.

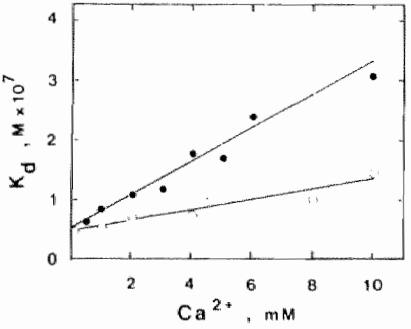

FIGURE 3: Summary of dissociation constants (K_d) for ^{125}I-factor Va LC-phospholipid binding (○) and factor Va-phospholipid binding (●) at different calcium concentrations. The dissociation constants calculated from double reciprocal plots (see e.g., Figure 2) are plotted versus added calcium. The phospholipid vesicle composition and phospholipid concentration are as given in Figure 2.

the negatively charged phospholipid polar head group by calcium (Hauser et al., 1976) would have an effect on the dissociation constant rather than on maximum protein binding capacity. A factor Va binding experiment using 20% DOPS large-volume vesicles at different calcium concentrations is shown in Figure 2. Calcium ions have an effect on the dissociation constant of factor

Va phospholipid interaction and not on the maximum protein binding capacity. Inother words, factor Va and calcium ions compete for binding sites on the phospholipid vesicle. We were not able to determine K_d and n values for factor Va phospholipid interaction in the absence of exogenous Ca^{2+}, since under this condition loss of factor Va activity was observed. From a replot of apparent K_d values vs. calcium concentration (Figure 3) it was calculated that in the absence of exogenous calcium the dissociation constant for factor Va-phospholipid interaction equals 0.045 μM. Also shown in Figure 3 is the replot of apparent K_d values for factor Va LC membrane interaction versus calcium concentration. It is interesting to see that the factor Va LC membrane interaction shows a smaller sensitivity to calcium than factor Va membrane interaction.

Another explanation than ionic shielding for the observed calcium effect must be considered. Calcium ions can induce lateral phase separation and irreversible vesicle fusion or aggregation of phospholipids. However, if vesicle fusion and/or aggregation of phospholipid did occur, we would expect a major effect on maximum protein-binding capacity rather than an effect on the dissociation constant. When the order of addition was changed, the same binding parameters were obtained, which means that factor Va and factor Va LC binding to membranes is reversible.

Effect of pH on Factor Va and Factor Va LC Phospholipid Interaction

The effects of pH on protein-phospholipid interaction were studied for two different vesicle compositions: 20% DOPA and 20% DOPS. The procedures and method of data analysis are simlar to those in Figure 2. The results for phosphatidylserine show minor changes in K_d between pH 7 and pH 9 (Figure 4). In addition the protein-binding capacity of the phospholipid vesicle did not vary in this pH range. It is apparent that there are no groups involved in protein-phospholipid interaction which ionize between pH 7 and pH 9. This is partially expected from the ionization groups found in phosphatidylserine (Hauser et al., 1976). It also implies that no groups in factor Va LC are involved in protein-lipid interaction which substantially ionize between pH 7 and pH 9. This observation is consistent with an isoelectric point of factor Va LC higher than 9.0.

In case of phosphatidic acid, pH has a major effect on the dissociation

constants of protein-phospholipid interaction. The protein-binding capacity of the vesicle did not vary between pH 7 and pH 9. In addition, the protein-binding affinities determined at pH 9 are similar to those of phosphatidylserine. These results indicate that monovalent phosphatidic acid, $pK_2 \sim 7$, has a reduced efficacy in factor Va and factor Va LC binding as compared to divalent phosphatidic acid and phosphatidylserine. Taking these results together, it appears that surface charge density is of prime importance for factor Va-phospholipid interaction.

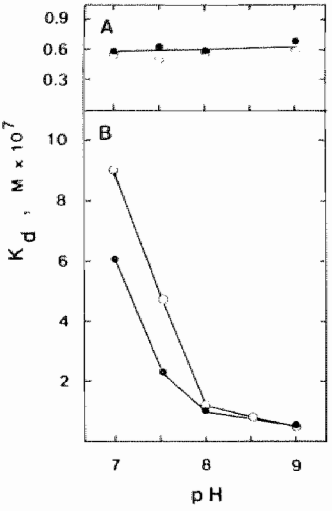

FIGURE 4: Effect of pH on the dissociation constants (K_d) of factor Va and ^{125}I-factor Va-LC phospholipid interaction. The dissociation constants were estimated from double reciprocal plots shown in Figure 2. Buffer used was 50 mM Tris, 100 mM NaCl, 0.2 mM $CaCl_2$, 1 mg ovalbumin/ml (pH values 7-9). The two vesicle compositions are 20% DOPS (panel A, 20 μM) and 20% DOPA (panel B, 25 μM). The dissociation constants for ^{125}I-factor Va LC-phospholipid binding (O) and factor Va-phospholipid binding (●) at varying pH are shown.

Effect of Ionic Strength on Factor Va-Phospholipid Interaction

Sensitivity to ionic strength is considered to be indicative for electrostatic interaction. Therefore we studied the effect of NaCl concentration on factor Va-phospholipid interaction. Binding experiments were performed using large-volume vesicles of 20% DOPS (30 μM) in 50 mM Tris, 1 mM $CaCl_2$, 1 mg ovalbumin/ml (pH 7.5) and varying NaCl concentrations. The binding parameters are shown in Table III. As anticipated for an ionic interaction, ionic strength has a major effect on the binding affinity. Since the maximum protein-binding capacity of the phospholipid vesicle is unaffected by ionic

strenght, it is apparent that factor Va competes with Na^+ for the same binding sites. Consistent with the 10^3-fold higher affinity of Ca^{2+} for phosphatidylserine than that of Na^+ (Hauser et al., 1976) is the difference in Ca^{2+} and Na^+ concentration required to compete with factor Va for phospholipid-binding sites. It further indicates that charge neutralization of the phospholipid surface by "screening" or "binding" of monovalent cations or divalent cations, respectively, to the surface (Puskin & Martin, 1979), is accompanied by the very same modulation of factor Va-membrane interaction.

Table III: Factor Va-Phospholipid[a] Binding to Vesicles at Varying Ionic Strenght

[NaCl](M)	K_d(M)	n[b]
0.05	2.2×10^{-8}	80
0.1	6.0×10^{-8}	73
0.5	32.0×10^{-8}	83

[a] 20% DOPS/80% DOPC

[b] Phospholipid to protein ratio at saturation.

DISCUSSION

At present, is is not possible to ascertain how factor Va-phospholipid interaction occurs. It has been suggested that factor V and factor Va bind to phospholipid vesicles by a nonelectrostatic interaction (Bloom et al., 1979). On the other hand, the possibility has been addressed that factor V contains some positively charged areas responsible for the binding to negatively charged phospholipid (Subbaiah et al., 1976).

In this study it is demonstrated that RVV-V activated factor Va consists of an acidic and basic polypeptide. It is shown that the basic factor Va sub-

unit, which has a net positive charge below pH 9, binds to phospholipid, whereas the acidic factor Va subunit is unable to bind to phospholipid. It is apparent that intact tertiary factor Va structure is not required to form the phospholipid-binding region of factor Va, since the equilibrium constants for thrombin activated factor Va-, RVV-V activated factor Va- and basic factor Va subunit-phospholipid interaction are identical.

Factor Va and the basic factor Va subunit did not bind to phospholipid vesicles containing solely phospholipid in its zwitterion state (e.g., phosphatidylcholine). However, the affinities of the proteins for vesicles increase with phosphatidylserine content. With respect to the dependency of maximum protein-binding capacity of the vesicle on the mole fraction of phosphatidylserine, it has to be mentioned that this binding parameter is relative insensitive to changes in phosphatidylserine content.

Lateral phase separation of phosphatidylserine molecules in the membrane would be accompanied by a positive free energy. An interesting question is whether lateral phase separation, as the result of factor Va-phospholipid interaction, is a major factor to the observed increase in K_d as the phosphatidylserine content decreases. Further studies are required to make this clear.

The initial question we addressed in this study is, does factor Va directly interact with phosphatidylserine molecules in the membrane. Several pieces of evidence indicate that this is the case. Competition for the factor Va and basic factor Va subunit binding site on the phospholipid vesicle come from divalent cations (e.g., Ca^{2+}). This is clearly demonstrated by the linear relationship between the apparent K_d for factor Va and basic factor Va subunit-membrane interaction and calcium concentration (see Figure 3). Since Ca^{2+}-phosphatidylserine interaction is an ionic process, it is feasable to conclude that phosphatidylserine molecules are directly involved in an electrostatic factor Va-membrane interaction. Additional evidence came from pH and ionic strenght studies. The effects of phospholipid charge neutralization is consistent with the proposed electrostatic protein-phospholipid interaction. That is, protonation of divalent phosphatidic acid results in a decrease in affinity of factor Va, whereas maximum protein-binding capacity is unaffected. Apparently, the pK values of the groups in the protein involved in protein-lipid interaction did not allow to establish charge neutralization of the protein as a major factor to protein-lipid binding in the pH

range studied. Further studies will be required to demonstrate unequivocally that basic amino acid residues present in the basic factor Va subunit are involved in the interaction with acidic phospholipids in the membrane. The ionic strength studies reveal that factor Va-phospholipid interaction has to be viewed primarily as an ionic process. However, hydrophobic contacts between factor Va and the hydrocarbon region of phospholipid cannot be eliminated on the basis of our data.

After this work was completed, Pusey et al. (1982) reported the phospholipid-binding properties of factor V. These authors also provide evidence for an ionic interaction between protein and phospholipid. However, their K_d values ($\sim 10^{-10}$ M) for factor Va and factor V-phospholipid interaction differ significantly. Whereas the light scattering technique used by Pusey et al. (1982) requires a number of assumptions, our method is direct. But for the moment, a straight-forward explanation for the discrepancy in binding data cannot be given.

From a mechanistic stand point, several interesting questions arise. Since factor Va shows the characteristics of an extrinsic membrane protein which requires a direct interaction with phosphatidylserine molecules in the membrane, factor Va probably competes with prothrombin, factor X, factor Xa and factor V for membrane-binding sites. Whether or not this might affect the kinetics of prothrombin activation will depend on a variety of parameters, like the number of available phospholipid-binding sites (e.g., phosphatidylserine molecules), the respective protein concentrations and specific high affinity protein-protein interactions [e.g., factor Va-factor Xa complex formation (Lindhout et al., 1982)].

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CHAPTER IV

THE EFFECT OF FACTOR Va ON MEMBRANE DYNAMICS OF MIXED PHOSPHOLIPID VESICLES AS PROBED BY FLUORESCENCE DEPOLARIZATION OF DIPHENYLHEXATRIENE

Piet van de Waart, H. Coenraad Hemker and Theo Lindhout

The influence of factor Va and calcium ions on the lipid dynamics of mixed phospholipid vesicles was monitored by steady-state fluorescence depolarization, using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent lipid probe. Two different lipid mixtures were studied: DMPS/DMPC, in which the neutral lipid is the lowest melting component and DMPS/DPPC, in which the neutral lipid is the highest melting component. Calcium ions and factor Va showed similar effects on the temperature dependent depolarization of DPH in vesicles: a) For both lipid mixtures there was no effect on the polarization value in the gel state of the membrane, b) 3 mM Ca^{++} increased the transition temperature (T_m) of 40% DMPS/60% DMPC and 40% DMPS/60% DPPC by 3 ^{o}C and 5 ^{o}C, respectively, while factor Va caused a minor increase in T_m of 1 ^{o}C and 2 ^{o}C, respectively and c) factor Va and Ca^{++} increased the polarization value in the liquid-crystalline state of both mixtures by about 0.05 units. The calcium effect was reversed upon the addition of EDTA, while the factor Va effect was not. The increase in polarization closely correlated with the amount of phospholipid-bound factor Va. The affinity of factor Va for phospholipid mixtures that contained PS was found to be dependent on the physical state of the membrane. Factor Va binds poorly to 20% DMPS/ 80% DMPC and 40% DMPS/ 60% DPPC in their gel state. Our results indicate that factor Va perturbs the membrane in the liquid-crystalline state by solidification of the PS molecules that interact with factor Va.

INTRODUCTION

Prothrombin and factor X(a) form complexes with negatively charged phospholipid (Lim et al., 1977) and appear to perturb the membrane, so as to cause lateral phase separation for membranes containing less than 20% of negatively charged phospholipid (e.g. phosphatidylserine) (Mayer & Nelsestuen, 1983).

Phospholipid binding of factor Va also requires negatively charged phospholipid. The interaction between factor Va and negatively charged phospholipid is electrostatic, where positively charged amino acid residues of factor Va, present in the 80,000 dalton polypeptide, interact with the phosphate of the polar phospholipid head groups (van de Waart et al., 1983). Light scattering studies have indicated that 25-30 phosphatidylserine residues interact with one factor V(a) molecule (Pusey et al., 1982). However, hydrophobic interactions between factor Va and the hydrophobic core region of phospholipids could not be ruled out.

In an attempt to improve our understanding of how factor Va interacts with membranes, we investigated its perturbating effect on phospholipid bilayers. In this paper, we present the results on the effects of factor Va on the thermotropic behavior of phospholipid as detected by fluorescence depolarization. Sonicated single lamellar vesicles and large-volume unilamellar vesicles prepared from DMPC and DMPS and DPPC and DMPS were used. Furthermore, we investigated factor Va-binding to phospholipids as a function of the physical state of phospholipids.

MATERIALS AND METHODS

1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes, Inc. 1,2-Dimyristoyl-sn-glycero-3-phosphoserine (DMPS) was prepared from 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) by enzymatic synthesis as described by Comfurius & Zwaal (1977). DMPC and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were from Sigma.

Single bilayer vesicles were prepared according to the method described by de Kruijff et al. (1975) by sonication for 10 min in 50 mM Tris, 100 mM

NaCl, pH 7.5 at 10 °C above the phase transition of the lipids, using a MSE Mark II ultrasonic desintegrator set at 7 μ peak to peak amplitude.

Large-volume vesicles were prepared by a modification (van de Waart et al., 1983) of the method described by Daemer & Bangham (1976). The composition of the vesicles is given as mole/mole ratios.

Factor Va was prepared and quantitated as previously described (Lindhout et al., 1982).

Mixtures of protein and large-volume vesicles were incubated for 10 min, followed by centrifugation at 30,000xg for 25 min. The indicated temperatures were maintained throughout the procedure. To determine phospholipid-bound factor Va, factor Va was estimated before and after centrifugation (van de Waart et al., 1983). Within the temperature range studied, no significant factor Va activity was lost.

DPH, dissolved in tetrahydrofuran (0.25 mg/ml), was added in a small volume, at a ratio of one probe molecule per 200 phospholipid molecules into vortexing vesicle suspensions. Nitrogen was then gently bubbled through the solution for 5 min, followed by a 30 min incubation period. The procedure was carried out at 10 °C above the phase transitions of the phospholipid mixtures.

Steady-state fluorescence depolarization experiments were performed on a thermostated Aminco-Bowman spectrofluorometer with polarizers (Glan Thompson prisms) in the excitation and emission beams (Shinitzky & Barenholz, 1978). The technique is based on determination of the rotational motion of the probe molecule. This motion is characterized by the polarization parameter, P, as defined by:

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

or the anisotropy parameter:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) = (2P)/(3-P)$$

where $I_{\parallel}$ is the fluorescence intensity parallel to and $I_{\perp}$ is the intensity perpendicular to the plane of polarization of the excitation beam. Unlike the polarization parameter (P), the anisotropy (r) is an additive parameter.

The excitation and emission wavelengths were set to 360 and 430 nm, respectively, and the relative intensities for the four combinations of

vertically and horizontally polarized excitation and emission beams were recorded in the ratio mode to eliminate source intensity fluctuations. Instrument output was displayed on a digital voltmeter. The different intensities (I) in the four combinations are denoted as follows:

emission polarizer	excitation polarizer	
	90^{o}	0^{o}
90^{o}	$I^{*}_{\parallel}$	$I_{\perp}$
0^{o}	$I^{*}_{\perp}$	$I_{\parallel}$

The steady state emission polarizations were then calculated as:

$$P = (I_{\parallel} - I_{\perp}.G) / (I_{\parallel} + I_{\perp}.G)$$

where the grating transmission factor $G = (I^{*}_{\perp}/I^{*}_{\parallel})$ represents a correction for the depolarization effect of grating monochromators (Chen & Bowman, 1965).

There was no depolarization due to light scattering since dilution of vesicles labeled with DPH had no effect on the fluorescence depolarization. Light scattering of the vesicle preparations in the absence of probe was never more than 5% of the fluorescence intensities. All fluorescence measurements were performed using a buffer containing 50 mM Tris, 100 mM NaCl, pH 7.5. Cuvette temperature was monitored during the experiments with a thermistor immersed in the cuvette.

The reproduced curves were measured at increasing temperature; before this, the samples were incubated for 5 min at 5 ^{o}C above the T_m. At each temperature measurements were made until stable values were obtained. The P values are the mean of four measurements. The standard error of the mean of four in a typical experiment appeared to be 0.005. Hysteresis associated with the temperature transitions were not found. No differences were observed between heating and cooling curves. The transition temperature (T_m) is defined as the midpoint of the range of maximal change in fluorescence depolarization.

RESULTS AND DISCUSSION

Fluorescence Depolarization as a Function of Temperature for Vesicles of Different Composition

Previous studies have shown that factor Va binds only to phospholipid mixtures that contain negatively charged phospholipids, e.g. phosphatidylserine (Bloom et al., 1979; Pusey et al., 1982; van de Waart et al., 1983). Phase separation as the result of the binding of other water-soluble basic proteins or Ca^{++} to negatively charged phospholipids has been reported (Ohnishi & Ito, 1974; Jacobson & Papahadjopoulos, 1975; Boggs et al., 1977; Hoekstra, 1982). Boggs et al. (1977) and Mayer & Nelsestuen (1981, 1983) reported a protein-induced shift in transition to a lower temperature if the acidic lipid is the higher melting component and to a higher temperature if the acidic lipid is the lower melting component. They suggested that the changes in T_m are due to clustering of the acidic phospholipid molecules, which enriches the bulk membrane in neutral lipid.

Because of its high sensitivity combined with its simplicity and clearly demonstrated utility, we have chosen the steady-state fluorescence depolarization technique to investigate factor Va-induced perturbations on phospholipid bilayers.

In order to allow interpretations of the effect of factor Va on structural reorganization in the membrane, we have studied the thermotropic behavior of two sets of phosphatidylserine (PS) and phosphatidylcholine (PC) containing mixtures, such that in one set PS is the higher melting component, while in the other it is the lower melting component: i.e. DMPC/DMPS and DPPC/DMPS, respectively.

To our knowledge, no data are available on the thermotropic behavior of these mixtures as probed by fluorescence depolarization of DPH embedded in these vesicles. In accordance with published data (Comfurius & Zwaal, 1977, Lentz et al. 1976) the observed transition temperatures were 35 ^{o}C and 22 ^{o}C for DMPS and for DMPC, respectively (Figure 1). As pointed out by Lentz et al. (1976), DPH is equally distributed between gel and liquid crystalline phases in the temperature range of the transitions where both phases coexist. Therefore, the measured anisotropy, r, of DPH would be a simple average of the anisotropy of the probe in each phase.

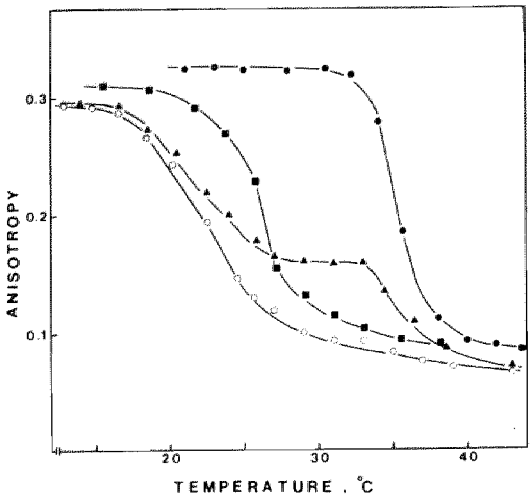

FIGURE 1: Steady state anisotropy at varying temperature for different lipid vesicles: (○) 100% DMPC; (●) 100% DMPS; (▲) equal amounts of vesicles of 100% DMPS and 100% DMPC; (■) vesicles of mixed lipids of 40% DMPS/60% DMPC. The lipid concentrations was $1.0x10^{-4}$ M.

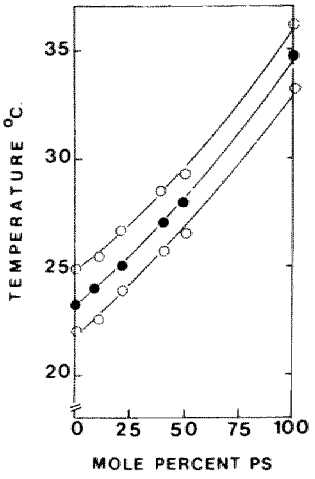

FIGURE 2: Plot of the transition temperatures as a function of the DMPC/DMPS ratio: (●) midpoint of transition, the two lines with the open circles depict the temperature range of transition.

This is clearly demonstrated for a mixture comprised of equal amounts of small vesicles composed of DMPC and similar vesicles composed of DMPS (Figure 1). The curve shows two transitions: one characteristic of pure DMPC, the other of pure DMPS. The anisotropy value (0.174) at 30 °C is approximately the simple average of the anisotropy of gel state DMPS (0.312) and the anisotropy of liquid DMPC (0.080).

Mixed vesicles that are comprised of 60% DMPC and 40% DMPS showed a single transition at 27 °C (Figure 1). This indicates that the two phospholipids mix both in the gel and liquid-crystalline state. Since a plot of T_m against given compositions does not deviate significantly from linearity, it

is apparent that the two phospholipids are randomly mixed in bilayers of small unilamellar vesicles (Figure 2). Whereas 100% DPPC showed a transition at 41 ^{o}C, mixtures of 20/80 and 40/60 (mol/mol) DMPS/DPPC showed a single transition temperature of 37.5 ^{o}C and 36 ^{o}C, respectively. This indicates that DMPS and DPPC also exhibit miscibility in both phases.

Effect of Factor Va on Fluorescence Depolarization of DPH Embedded in Sonicated Vesicles Composed of DMPS/DMPC amd DMPS/DPPC.

The effect of factor Va on the thermotropic behavior of sonicated vesicles composed of 20% DMPS/80% DMPC or 40% DMPS/60% DMPC is shown in Figure 3A and 3B, respectively. It is clear that: (i) there is a small increase of the T_m in the presence of factor Va, and (ii) the depolarization of DPH is reduced above the T_m of the mixed lipid bilayer, while it is not altered by factor Va when the lipids are in the gel state. Similar results were obtained with sonicated vesicles composed of 20% DMPS/80% DPPC or 40% DMPS/60% DPPC (Figures 3C and 3D, respectively).

It is apparent that factor Va has a small effect on the cooperative behavior of the mixed lipid phase transition. In both lipid mixtures whether the neutral lipid is the lower or higher melting component, factor Va increased the T_m of DMPS/DMPC and DMPS/DPPC by 1 and 2 ^{o}C, respectively. This is in contrast with the results reported by Mayer & Nelsestuen (1983). They found that addition of factor V increased the T_m of brain PS-DPPC (20/80) by a T_m shift proportional to the amount of phospholipid-bound factor V. Moreover, addition of factor V decreased the T_m of a phospholipid mixture that consisted of 30% DPPA (T_m = 58 ^{o}C) and 70% DMPC (T_m = 22 ^{o}C). These authors monitored the phase transition by measuring the fluorescence intensities ($I^{*}_{//}$, see Materials and Methods) of 8-anilonaphithalene-1-sulfonic acid (ANS) embedded in the vesicle. Although their experimental approach differed from ours, we have no explanation why they, in contrast to our findings, could observe a decrease in T_m by utilizing a phospholipid mixture in which the neutral phospholipid was the lower melting component. Apparently, this is not due to the poor miscibility of the two lipids, because we found that the addition of factor Va to vesicles composed of 80% DOPC (T_m = -18 ^{o}C and 20% DMPS (T_m = 35 ^{o}C) did not decrease, but slightly increased the T_m (data not shown).

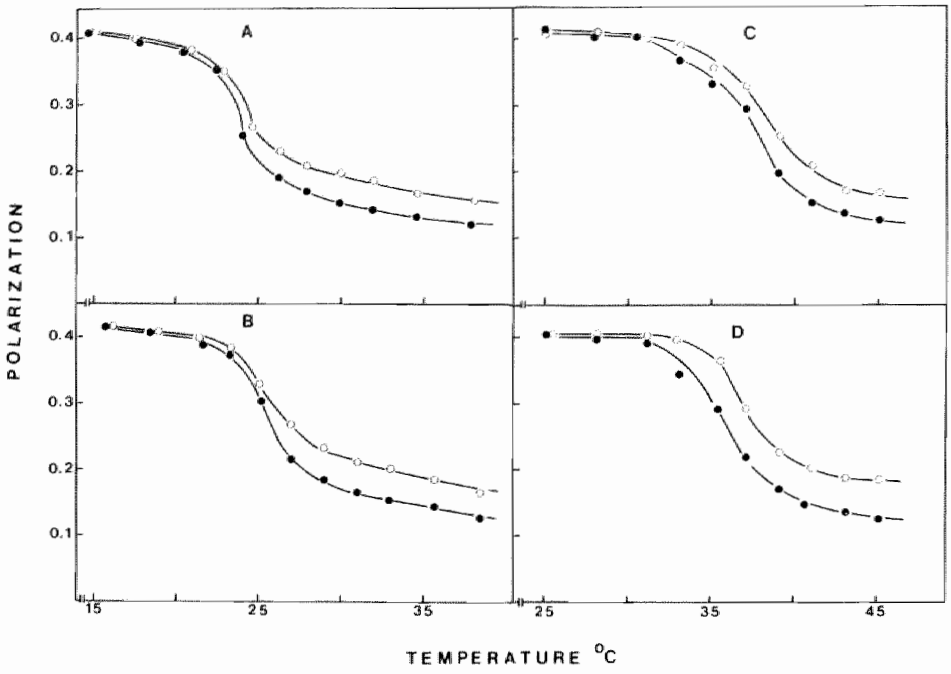

FIGURE 3: Effect of factor Va on the temperature dependend polarization of different lipid vesicles. The mole/mole ratios are given between brackets. A, DMPS/DMPC (20/80); B, DMPS/DMPC (40/60); C, DMPS/DPPC (20/80); D, DMPS/DPPC (40/60). Closed and open circles: in the absence and presence of $2.0x10^{-7}$ M factor Va respectively. The lipid concentration was $5.0x10^{-5}$ M.

Calcium ions, like factor Va, interact electrostatically with negatively charged phospholipids and can trigger alterations in the thermotropic behavior of membranes (Newton et al., 1978; Ohnishi & Ito, 1974; Hauser et al., 1976; van Dijck et al., 1978). Therefore, it was interesting to compare the effects of Ca^{++} on the thermotropic behavior of lipid mixtures with those found for factor Va.

Figure 4 shows the polarization curves as a function of temperature for DMPS/DMPC and DMPS/DPPC vesicles, both 40/60 mole/mole ratio, in the absence and presence of 3 mM Ca^{++}. Whether when PC was the lowest or highest melting component, Ca^{++} induced the same effects: (i) no effect on the polarization

in the gel state, (ii) an increase in T_m by 3 °C and 4 °C, respectively and (iii) a reduced depolarization in the liquid-crystalline state. The addition of EDTA to the calcium-lipid mixtures completely reversed the Ca^{++}-induced alterations (data not shown), while the addition of EDTA to factor Va-lipid mixtures had no effect.

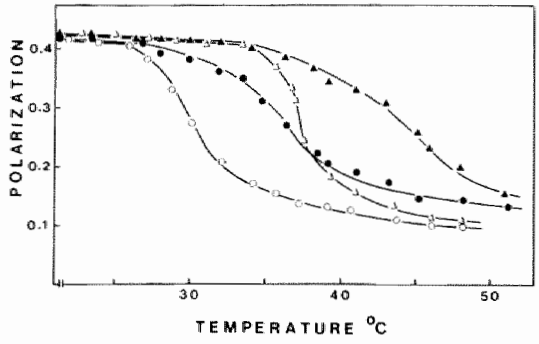

FIGURE 4: Effect of Ca^{2+} on the temperature dependent polarization of vesicles composed of DMPS/DMPC (circles) and DMPS/DPPC (triangles), both 40/60 mole/mole ratio. Open and closed symbols: in the absence and presence of 3 mM $CaCl_2$, respectively. The lipid concentration was $5.0x10^{-5}$ M.

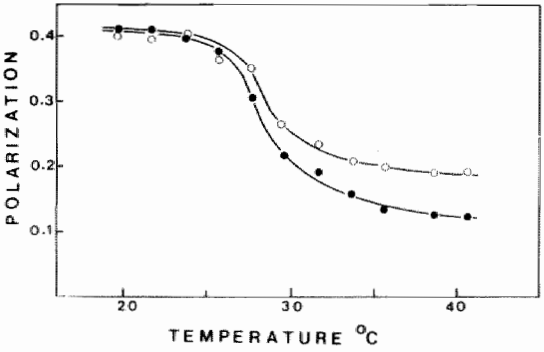

FIGURE 5: Effect of factor Va on the temperature dependent polarization of large volume vesicles composed of 40% DMPS and 60% DMPC. Closed and open symbols: in the absence and presence of $4.0x10^{-7}$ M factor Va, respectively. The lipid concentration was $1.0x10^{-4}$ M.

We conclude that neither a Ca^{++}- nor a factor Va-induced phase separation could be demonstrated. Because factor Va and Ca^{++} decreased the polarization of DPH embedded in vesicles in their liquid-crystalline state, it can be concluded that factor Va does induce a higher order of the lipid molecules. It has been demonstrated that Ca^{++} or positively charged proteins can condense and solidify negatively charged phospholipids in the bilayer, when the phospholipid is in a pure state or in a mixture with a neutral lipid at constant temperature (Verkleij et al., 1974).

Since DPH is equally distributed between gel and liquid-crystalline phases, it is likely that the polarization value is the average of the polarization value of solidified phosphatidylserine molecules that interact with factor Va and that of the bulk phospholipid mixture. This would be accompanied by an enrichement of the bulk phospholipid with neutral lipid and in that way might also affect the T_m. Whether this can be detected will depend on the magnitude of each of the contributions to the mean depolarization value. Time-resolved anisotropy measurements are planned to approach this problem.

To ascertain that the effect on the polarization value was due to binding of factor Va to phospholipid, we performed titration experiments as well as binding experiments of factor Va onto large-volume vesicles that consist of DMPS/DMPC and DMPS/DPPC at temperatures above the T_m.

These vesicles show properties that are well comparable to those of the smaller vesicles used before. The T_m of large-volume vesicles composed of 40% DMPS/60 % DMPC was found to be 27.5 ^{o}C (Figure 5). Sonicated vesicles of identical composition exhibit a T_m of 25.5 ^{o}C. Similar differences between sonicated vesicles and liposomes (Lentz et al., 1976) or unilamellar vesicles of different size (Lichtenberg et al., 1981) have been reported. The T_m of large-volume vesicles composed of 40% DMPS/60% DPPC was found to be 1 ^{o}C higher than that of sonicated vesicles of identical composition. As shown in Figure 5, factor Va perturbs the membrane of large-volume vesicles in a similar fashion to that observed for sonicated vesicles.

Figure 6A shows that the change in degree of polarization of DPH embedded in 40%DMPS/60% DMPC vesicles in the liquid state is directly proportional to the amount of phospholipid-bound factor Va. The amount of bound factor Va and the degree of polarization of DPH embedded in vesicles composed of 40% DMPS/60% DPPC in the liquid-crystalline state is shown in Figure 6B. Again, the P value increased concomitant with protein binding. The binding experiments were carried out in the presence of 0.3 mM Ca^{++}. Omitting the calcium resulted in a 30% loss of factor Va activity during the course of the experiment. Ca^{++} at a concentration of 0.3 mM affects the depolarization of DPH embedded in 40% DMPS/60% DMPC vesicles. At 37 ^{o}C the P values were 0.175 and 0.250 in the absence or presence of 0.3 mM Ca^{++}, respectivly. Calcium, 0.3 mM, also increased the P value of vesicles comprised of 40% DMPS/60% DPPC from 0.184 to 0.314, as determined at 40 ^{o}C.

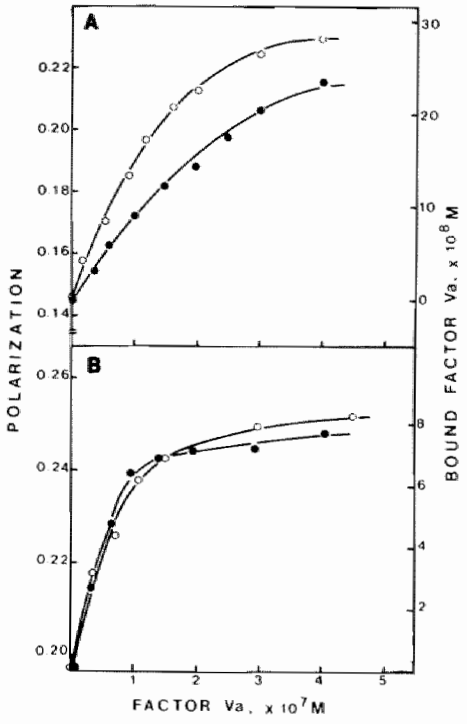

FIGURE 6: Polarization changes induced by factor Va binding to large volume vesicles. The amount of phospholipid-bound factor Va (closed symbols) and polarization (open symbols) are depicted as a function of the amount of added factor Va. The measurements were performed at 37 °C for DMPS/DMPC (panel A) and at 40 °C (for DMPS/DPPC (panel B). The PS/PC ratio was 40/60, and the lipid concentration was 1.0×10^{-4} M.

Although Ca^{++} affects the membrane dynamics of these mixed phospholipid vesicles, it probably will not significantly affect the binding of factor Va. Previous work has shown that the affinity of factor Va for negatively charged phospholipids is much higher than that of Ca^{++}. Factor Va strongly competes with Ca^{++} and so the binding data will be close to those obtained in the absence of calcium (van de Waart et al., 1983).

An other interesting point is that factor Va had no effect on the polarization of DPH embedded in lipids in the gel state. The absence of a factor Va-induced reorganisation of the phospholipid chains in the gel state, almost precludes hydrophobic interactions and protein penetration in the gel state of the bilayer (Faucon et al., 1983; Jähnig, 1979). Since a freezing-out phenomenon was not observed, it is apparent that a hydrophobic interaction between factor Va and lipid in the liquid-crystalline state of the membrane can also be precluded. In addition, the question arised whether the binding of factor Va depends on the physical state of the phospholipids.

Figure 7 shows the binding of factor Va to large-volume phospholipid vesicles composed of 20% DMPS/80% DMPC or 20% DMPS/80% DPPC as a function of

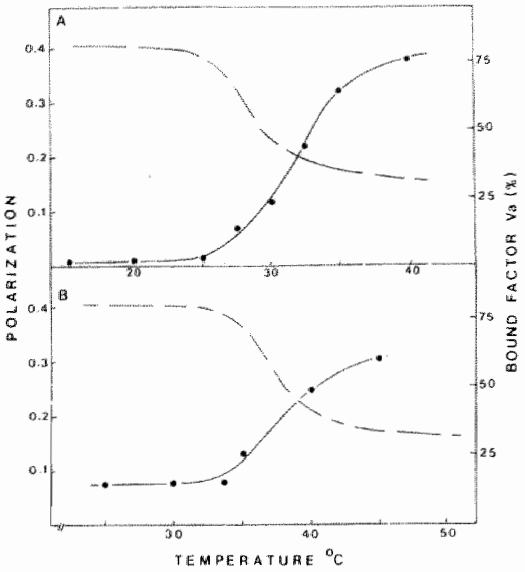

FIGURE 7: Factor Va binding to vesicles as function of temperature. The closed and the open symbols represent the respective binding and polarization for: panel A, $5.0x10^{-5}$ M DMPS/DMPC (20/80) and $3.0x10^{-7}$ M factor Va; panel B, $1.0x10^{-4}$M DMPS/DPPC (40/60) and $3.0x10^{-7}$ M factor Va.

the physical state of the membrane. It is clearly demonstrated that the amount of phospholipid-bound factor Va increased concomitant with the decrease in ordering of the aliphatic chains as probed by the fluorescence depolarization of DPH. The binding experiments were carried out at 0.3 mM Ca^{++}, while the depolarization of DPH was measured in the absence of Ca^{++}. It is interesting to see that the binding correlates with the transition of the lipid in the absence of Ca^{++} and not with the Ca^{++}-induced perturbation of the membrane. It is apparent that this is due to the competition between factor Va and Ca^{++}.

If the binding of factor Va to phospholipids governs the activity of the prothrombinase complex, it explains why the optimal procoagulant activity of lipid mixtures was observed at the T_m of the lipids in the absence of Ca^{++} and did not coincides with the T_m in the presence of the Ca^{++} concentration used, i.e. 3.5 mM (Tans et al., 1979).

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CHAPTER V

THE INTERACTION OF PROTHROMBIN WITH FACTOR VA-PHOSPHOLIPID COMPLEXES

Piet van de Waart, H.Coenraad Hemker and Theo Lindhout

The effects of factor Va and the phospholipid-binding fragment of factor Va (factor Va LC, M_r=80,000) on the binding of prothrombin, factor X and factor Xa to phospholipid vesicles are reported. Equilibrium binding experiments were performed utilizing large-volume vesicles, which can be removed from the bulk solution by centrifugation. Factor Va decreased the dissociation constant of the prothrombin-phospholipid complex 50 fold, from 2.0 x 10^{-7} M to 4.0 x 10^{-9} M. For the factor X-phospholipid complex the decrease was 60 fold (1.8 x 10^{-7} M to 3.0 x 10^{-9} M) and for factor Xa 160 fold (1.6 x 10^{-7} M to 1.0 x 10^{-9} M). The ratios of moles protein bound to moles total added factor Va at saturation of phospholipid-bound factor Va, indicate an 1:1 stoichiometric complex of either factor Xa, factor X or prothrombin and phospholipid-bound factor Va. In the presence of factor Va LC, the dissociation constants of factor Xa- and prothrombin-phospholipid complexes were increased, while the maximal protein-binding capacities of the vesicles were not affected by factor Va LC. The data suggest a competitive interaction between factor Xa and factor Va LC binding as well as between prothrombin and factor Va LC binding at the phospholipid surface. From this it is concluded that the phospholipid-binding fragment of factor Va alone, does not serves as the binding site for interactions of factor Xa and prothrombin with factor Va.

INTRODUCTION

Conversion of prothrombin into thrombin by factor Xa is most efficient in the presence of factor Va and membranes containing acidic phospholipids. Rosing et al. (1980) demonstrated that phospholipids lower the K_m for prothrombin, while factor Va increases the V_{max} of thrombin formation.

Equilibrium binding experiments employing various techniques have indicated that the interaction of prothrombin and factor Xa with acidic phospholipid is mediated by calcium ions (Nelsestuen & Lim, 1977; Mayer et al., 1983). However, factor Va associates with membranes directly by interacting with the polar head groups of the acidic phospholipids (van de Waart et al, 1983a).

It is well appreciated that a phospholipid surface is required for a proper assembly of the proteins involved in prothrombin activation. The formation of the catalytic unit, a stoichiometric (1:1) factor Xa-factor Va complex with a dissociation constant of 3×10^{-9} M, is stimulated by acidic phospholipids, resulting in a phospholipid-bound factor Xa-factor Va complex with a dissociation constant of approximatly 10^{-10} M (Nesheim et al., 1979; Lindhout et al., 1982).

Localization of the prothrombinase complex on a phospholipid surface *per se*, as facilitated by factor Va, does not explain the effect of factor Va on the catalytic efficiency of factor Xa. To that end, a role of factor Va in the formation of enzyme-substrate complex was proposed (Esmon & Jackson, 1974; Rosing et al., 1980). However, quantitative studies of the interaction of prothrombin with factor Va-phospholipid complexes have not been reported.

In a recent report, Tracy & Mann (1983) described experiments indicating that the interaction of factor Va with the platelet surface is mediated primarily through factor Va LC, the 80,000 dalton subunit of factor Va. This observation is consistent with our results using artifically prepared phospholipid membranes (van de Waart et al., 1983a). Tracy & Mann (1983) suggested that the 80,000 dalton subunit of factor Va alone provides the binding-site for the interaction of factor Xa with platelet-bound factor Va. If artificially prepared phospholipid membranes are a suitable model for the biological membrane surface (platelets), then these results are not in agreement with our observation that the calcium-mediated interaction between

the subunits of factor Va is required for factor Xa binding (Lindhout et al., 1982).

In this paper, we describe experiments indicating that the binding of prothrombin to membranes containing acidic phospholipids is facilitated by factor Va. We further demonstrate that the phospholipid binding subunit of factor Va (M_r = 80,000) does not exhibit this effect but competes with factor Va, factor Xa and prothrombin for the same phospholipid binding sites (i.e. acidic phospholipids).

MATERIALS AND METHODS

Ovalbumin and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained from Sigma. All other chemicals were of analytical grade. Factor Xa and thrombin activity were determined using the chromogenic substrates N-benzoyl-L-isoleucyl-L-glutamyl-(piperidyl)-glycyl-L-arginine-p-nitroanalide hydrochloride (S2237) and H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroanalide dihydrochloride (S2238) (Kabi Diagnostics), respectively.

The following bovine clotting factors were prepared: prothrombin (Owen et al., 1974), thrombin (Rosing et al., 1980), factor V, factor Va and factor Va subunits (Lindhout et al., 1982), factor X (Fujikawa et al., 1972a) and factor Xa (Fujikawa et al., 1972b).

Functional molar concentrations of factor Va and factor Va subunits, factor Xa and prothrombin were estimated according to van de Waart et al. (1983a), van Dieijen et al. (1981) and Rosing et al. (1980), respectively.

1,2-Dioleoyl-sn-glycero-3-phosphoserine (DOPS) was prepared according to the method described by Comfurius & Zwaal (1977).

Large volume unilamellar vesicles were prepared by the ether injection method according to Deamer & Bangham (1976) as previously described (van de Waart et al., 1983a). Whenever a membrane composition is expressed as a percentage of acidic phospholipid, the remaining phospholipid is DOPC.

Phospholipid concentrations were determined by the method of Böttcher et al., (1961).

Binding of proteins to large-volume vesicles was quantitated by measuring the molar concentrations of the proteins prior to and after centrifugation

for 30 min at 30,000 g and 20 °C. Protein binding data were analysed by double-reciprocal plots of the concentrations of bound protein versus free protein (van de Waart et al., 1983a).

All binding experiments were carried out in a buffer containing 50 mM Tris, 100 mM NaCl, 0.5 mg ovalbumin/ml, 3 mM $CaCl_2$, pH 7.5.

RESULTS

Initial Experiments

Quantitative binding studies, employing various techniques, on the interaction of factor Xa with artificially prepared membranes as well as with platelets in the presence of factor Va have been reported (Nesheim et al., 1979; Tracy et al., 1981; Lindhout et al., 1982).

As yet, no such studies have been described on factor Va-prothrombin interaction at a phospholipid surface. A simple and direct binding technique, utilizing large-volume vesicles which can be removed from the bulk solution by centrifugation, is a valuable tool in the investigation of protein-protein interactions at a phospholipid surface (van de Waart et al., 1983a).

Using this technique, we found that the amount of factor Xa associated with vesicles containing 5% DOPS increased dramatically between 0 and 0.7 μM factor Va added up to a value approaching 100% of the added factor Xa (Figure 1). In the absence of factor Va, approximately 10% of total added factor Xa was associated with these vesicles. Vesicles containing 20% DOPS instead of 5%, have a relatively high affinity for factor Xa in the absence of factor Va and consequently show a less pronounced facilitation of the binding by factor Va.

The binding of prothrombin to vesicles containing 20% DOPS was also facilitated by factor Va (Figure 2). The maximal amount of prothrombin bound to the vesicles was obtained at a factor Va concentration of 0.3 μ M. At higher factor Va concentrations, a gradual decrease in prothrombin associated with the vesicles was observed.

The light-scattering technique cannot be used to establish competition between two proteins for the same phospholipid binding sites, whereas the binding technique used here can. This is seen from Figure 3.

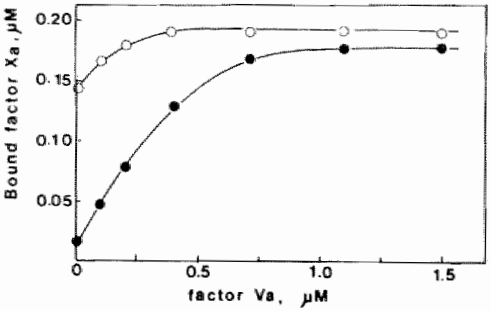

FIGURE 1: Binding of factor Xa to 5% DOPS (●) and 20% DOPS (○) vesicles in the presence of varying concentrations of factor Va. Binding measurements were made following a 10-min incubation. The factor Xa concentration was kept constant at 2.0 x 10^{-7} M. The phospholipid concentrations were either 2.0 x 10^{-4} M (5% DOPS) or 5.0 x 10^{-5} M (20% DOPS).

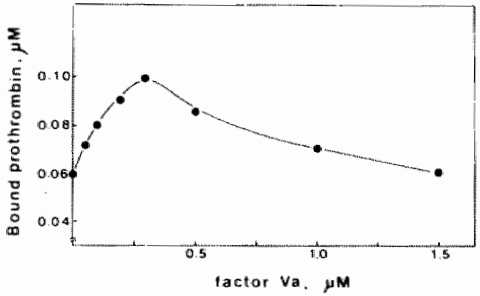

FIGURE 2: Binding of prothrombin to 20% DOPS vesicles in the presence of varying concentrations of factor Va. The prothrombin concentration was kept constant at 2.0 x 10^{-7} M. Phospholipid concentration was 1.0 x 10^{-4} M.

The phospholipid-binding properties of factor Xa were studied in the presence of various amounts of factor X. The double reciprocal plots of bound versus free factor Xa yield straight lines. The respective dissociation constants were 2.1 x 10^{-7} M, 4.2 x 10^{-7} M and 1.0 x 10^{-6} M for no factor X present, 1.5 x 10^{-7} M and 3.0 x 10^{-7} M of factor X, respectively. The maximal binding capacity of the vesicles for factor Xa was not affected by factor X, indicating that at infinitely large free factor Xa concentration all factor X, was deplaced from the phospholipid surface. These results are consistent with a system of two ligands competing for one class of binding sites with no mutual interactions.

In a completely analogous way, the apparent dissociation constant of factor Va-phospholipid complexes increased with the factor Va LC concentration (Figure 4). The respective dissociation constants were 2.0 x 10^{-8} M, 5.0 x 10^{-8} M and 1.0 x 10^{-7} M for no factor Va LC present, 1.0 x 10^{-7} M and 2.0 x 10^{-7} M of factor Va LC, respectively. The competition between factor Va and

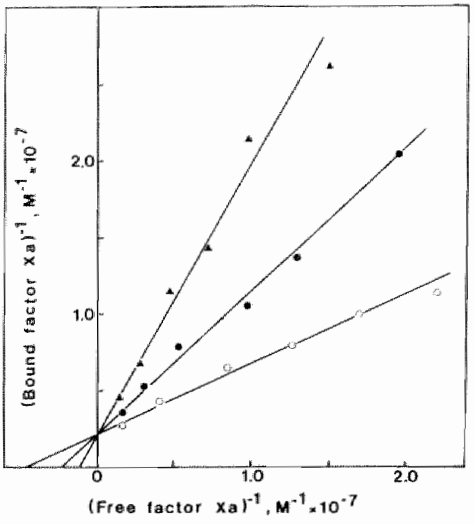

FIGURE 3: Double reciprocal plots of factor Xa binding to vesicles at varying amounts of factor X. The amounts of bound and free factor Xa were determined from reaction mixtures containing no factor X (○), 1.5 x 10^{-7} M (●) or 3.0 x 10^{-7} M (▲) of factor X, factor Xa (1.0 x 10^{-7} M to 1.0 x 10^{-6} M) and 2.5 x 10^{-5} M phospholipid (20% DOPS). Binding measurements were done as decribed in Materials and Methods.

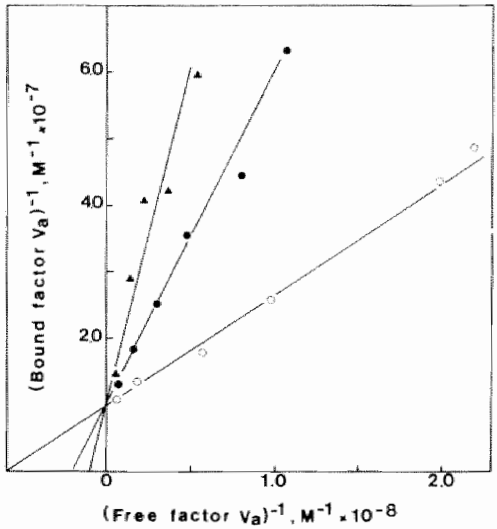

FIGURE 4: Double reciprocal plots of factor Va binding to vesicles at varying amounts of factor Va LC. The amounts of bound and free factor Va were determined from reaction mixtures containing no factor Va LC (○), 1.0 x 10^{-7} M (●) or 2.0 x 10^{-7} M (▲) of factor Va LC, factor Va (3.0 x 10^{-8} M to 3.0 x 10^{-7} M) and 1.0 x 10^{-5} M phospholipid (20% DOPS). Binding measurements were done as described in Materials and Methods.

factor Va LC for the phospholipid binding sites, confirm earlier findings that factor Va and factor Va LC have identical phospholipid-binding properties (van de Waart et al., 1983a).

Effect of Factor Va on Equilibrium Binding Data of Prothrombin-, Factor X-, and Factor Xa-Phospholipid Interactions

Figure 5A shows that in the absence of factor Va, the ratio of phospholipid-bound prothrombin to free prothrombin is constant. Apparently, the binding sites are in large excess over the prothrombin molecules. This is consistent with a dissociation constant of 2×10^{-7} M and a maximal prothrombin binding capacity of the vesicles of 2×10^{-7} M prothrombin per 4×10^{-5} M phospholipid (van de Waart et al., 1983a). As indicated by the changes in B/F values, the addition of amounts of factor Va, small as compared to the amount of binding sites and comparable to the amount of prothrombin added, yields two distinct classes of prothrombin binding sites.

Analysis of the binding data by double reciprocal plots of concentration of bound prothrombin versus concentration of free prothrombin showed the presence of high affinity and low affinity phospholipid binding sites with dissociation constants of 5×10^{-9} M and 1.5×10^{-7} M, respectively (Figure 5B).

It is apparent that the number of high affinity binding sites is proportional to the concentration of factor Va. It in fact equals approximately 90% of total added factor Va. Under the conditions of this experiment nearly 100% of added factor Va is associated with the phospholipids.

So the high affinity binding sites for prothrombin probably are the factor Va-phospholipid complexes. The high number of binding sites with a dissociaton constant of 10^{-7} M is provided by non-complexed phospholipid.

Decreasing the DOPS content of the vesicles increased the apparent dissociation constant of prothrombin interaction with phospholipid-bound factor Va, while the number of high affinity sites appeared not to be dependent on the DOPS content (Table I). This is consistent with the documented increase of the dissociation constants of both proteins with decreasing DOPS content in vesicles and with the observation that under the experimental conditions used in this study, the amount of phospholipid-bound factor Va is not significantly dependent on the DOPS content in vesicles.

For reasons of comparison, we investigated the effect of factor Va on factor X- and factor Xa- binding to vesicles in a similar set of experiments. As in the study with prothrombin, biphasic curves were obtained in all cases where factor Va was included in the reaction mixtures (data not shown).

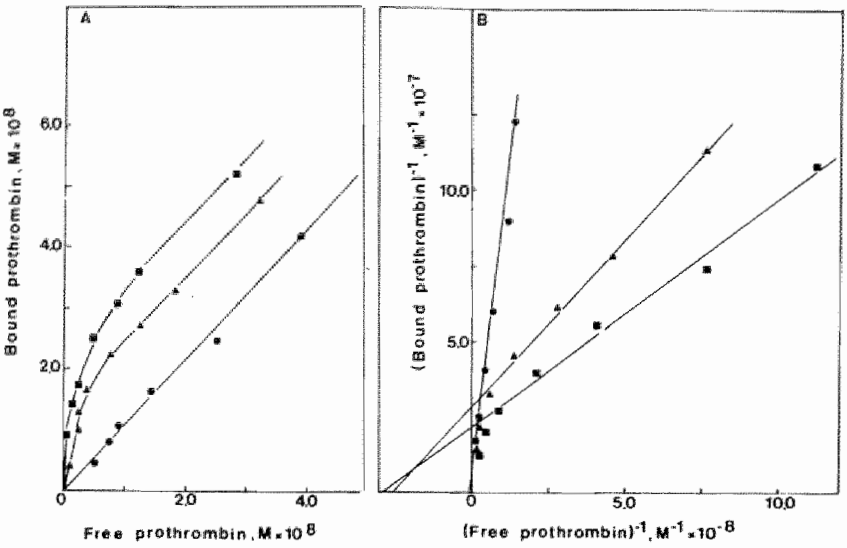

FIGURE 5: Binding of prothrombin to vesicles in the presence of factor Va. Panel A: the amounts of bound and free prothrombin were determined from reaction mixtures containing no factor Va (●), 3.0 x 10^{-8} M (▲) or 8.0 x 10^{-8} M (■) factor Va, varying amounts of prothrombin (0.1 x 10^{-7} M to 1.5 x 10^{-7} M) and 4.0 x 10^{-5} M phospholipid (20% DOPS). Panel B: double reciprocal plots of the binding data from A.

The respective binding parameters, obtained by linear extrapolation of the data points in the double reciprocal plot, that represent the high affinity interaction are shown in Table I, along with the prothrombin binding data. The binding parameters for the interaction between factor Xa and phospholipid-bound factor Va compare with previous reported binding parameters using sonicated vesicles (Nesheim et al., 1979; Lindhout et al., 1982).

As is also shown in Table I, the affinity of factor X for the phospholipids is increased by two orders of magnitude by the presence of factor Va. It is apparent that factor Xa binds more tightly to phospholipid-bound factor Va than does the proenzyme factor X; the binding constants are 1.1 x 10^{-9} M

Table I: Prothrombin-, Factor X- and Factor Xa-Factor Va Binding Interactions at a Phospholipid Surface

Varied component	Fixed component			Kd,app (M)	n[a]
	phospholipid		factor Va		
	% DOPS	conc. (M)	conc (M)		
Factor Xa	20	2.5×10^{-5}	0	1.6×10^{-7}	
	10	2.5×10^{-5}	0	4.0×10^{-7}	
	20	1.0×10^{-5}	5.0×10^{-9}	1.0×10^{-9}	0.96
	20	1.0×10^{-5}	3.0×10^{-8}	1.2×10^{-9}	0.66
	10	5.0×10^{-5}	6.0×10^{-8}	2.0×10^{-9}	0.73
Factor X	20	2.5×10^{-5}	0	1.8×10^{-7}	
	20	2.5×10^{-5}	5.0×10^{-8}	3.0×10^{-9}	1.10
Prothrombin	20	5.0×10^{-5}	0	2.0×10^{-7}	
	10	5.0×10^{-5}	0	4.0×10^{-7}	
	20	4.0×10^{-5}	3.0×10^{-8}	4.5×10^{-9}	1.2
	20	4.0×10^{-5}	8.0×10^{-8}	4.0×10^{-9}	0.62
	10	4.0×10^{-5}	3.0×10^{-8}	1.5×10^{-8}	0.86

[a] Ratio of moles protein bound to moles total added factor Va at saturation of phospholipid-bound factor Va

and 3.5×10^{-9} M, respectively. This is consistent with the observation that factor X is not as effective as factor Xa in decreasing the rate constant of dissociation of factor Va-phospholipid complexes (Pusey et al., 1982).

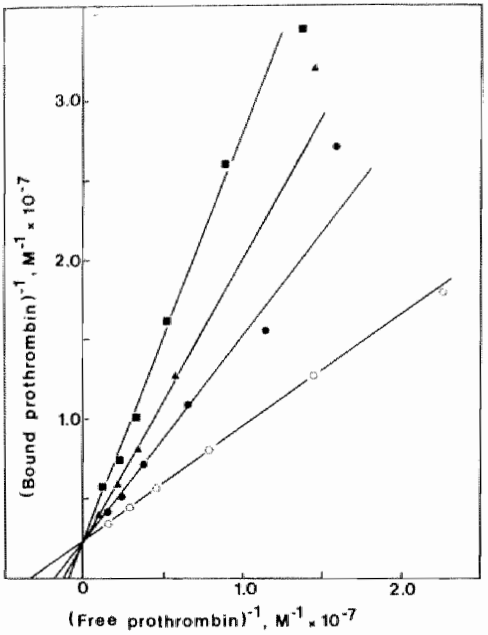

FIGURE 6: Double reciprocal plots of prothrombin binding to vesicles at varying amounts of factor Va LC. The amounts of bound and free prothrombin were determined from reaction mixtures containing no factor Va LC (○), 1.0 x 10^{-7} M (●), 3.0 x 10^{-7} M (▲) or 6.0 x 10-7 M (■) of factor Va LC, prothrombin (8.0 x 10^{-8} M to 1.2 x 10^{-6} M) and 1.0 x 10^{-4} M phospholipid (20% DOPS). Binding measurements were done as described in Materials and Methods.

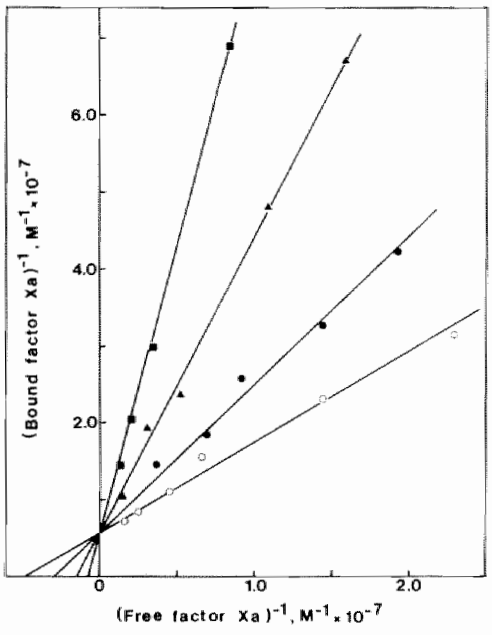

FIGURE 7: Double reciprocal plots of factor Xa binding to vesicles at varying amounts of factor Va LC. The amounts of bound and free factor Xa were determined from reaction mixtures containing no factor Va LC (○), 2.3 x 10^{-7} M (●), 3.0 x 10^{-7} M (▲) or 5.0 x 10^{-7} M (■) factor Va LC, factor Xa (7.5 x 10^{-8} M to 7.5 x 10^{-7} M) and 1.2 x 10^{-5} M phospholipid (20% DOPS). Binding measurements were done as described in Materials and Methods.

Association of Prothrombin and Factor Xa with Phospholipid Vesicles in the Presence of Factor Va LC

The association of prothrombin with vesicles containing 20% DOPS was determined in the presence of varying amounts of factor Va LC (Figure 6). Contrary to the effect observed with factor Va, dissociation constant of prothrombin-phospholipid complexes increased with increasing factor Va LC concentrations, while the phospholipid to prothrombin ratio at infinite free prothrombin concentration is independent of the amount of factor Va LC present. The relationship between the apparent dissociation constants of prothrombin-phospholipid complexes and factor Va LC concentration indicates that prothrombin and factor Va LC compete for phospholipid binding sites, i.e. acidic phospholipids. Furthermore, it can be concluded that factor Va LC alone does not serve as the binding site for interaction of prothrombin with factor Va.

Also for the binding of factor Xa to phospholipid vesicles in the presence of factor Va LC, a competition between factor Xa and factor Va LC for the phospholipid binding sites was demonstrated (Figure 7). It is apparent that the interaction between factor Va and factor Xa at the phospholipid surface is not mediated by factor Va LC.

DISCUSSION

With the use of the simple and direct technique of equilibrium binding on large volume vesicles, we undertook to further study the role of factor Va in the assembly and function of the prothrombinase complex. This paper describes as yet unreported equilibrium binding experiments on factor Va- prothrombin complex formation at a phospholipid surface. We also investigated the structure-function relationship between factor Va as well as its phospholipid-binding subunit (factor Va LC) and the other components of the prothrombinase complex, i.e. factor Xa and phospholipid, as well as the substrate of the prothrombinase complex, prothrombin.

Previous studies of factor Xa-, factor Va- and prothrombin- phospholipid complexes indicate that the proteins of the prothrombinase complex and the

substrate of the complex, associate with membranes exclusively through interaction of the proteins with the negatively charged polar head groups of the phospholipids (Pusey et al., 1982; Mayer et al., 1983 and references therein; van de Waart et al., 1983a). Clustering of acidic phospholipids in membranes of low PS content (< 20%) probably accompanies these protein-membrane association processes (Mayer & Nelsestuen, 1981).

Taking these observations together, it is easy to conceive that, depending on the respective protein-protein and protein-phospholipid affinities, coordinate and/or competitive binding interactions might occur between proteins at the phospholipid surface.

The present studies provide several examples of competitive as well as coordinate interactions which might be of importance for prothrombin activation. It is apparent that factor X and factor Xa compete for phospholipid binding sites (Figure 3). This is consistent with the following observations: 1) The dissociation constants of factor X- and factor Xa-phospholipid complexes were found to be identical (van de Waart et al., 1983a), 2) one phospholipid bound factor X and factor Xa molecule covers about 6 phosphatidylserine molecules (Mayer et al., 1983) and 3) factor Xa has a low affinity towards factor X (Link & Castellino, 1982).

Given the observations, that factor Xa binding to phospholipid is essential to prothrombin activation and the competitive binding interaction between factor X and factor Xa for phospholipid binding sites, the question arises whether the presence of a large excess of the proenzyme factor X over factor Xa might influence the conversion of prothrombin to thrombin at a phospholipid surface (platelets) by factor Xa. This question cannot be answered unless the interaction of factor Xa with the protein components of the prothrombin-prothrombinase complex are taken into account. To this end, it is of interest to observe that factor Va facilitates the binding of both factor X and factor Xa to the phospholipid surface by decreasing the dissociation constant in the same order of magnitude (60-fold and 160-fold, respectively, Table I). From these results, the notion arises that factor X might be able to remove factor Xa from factor Xa-factor Va complexes competitively and thus reduces the amount of functional phospholipid-bound enzyme. Preliminary data from kinetic studies on prothrombin activation have given support to this possibility (unpublished results).

Apart from its function in the assembly of the prothrombinase complex, factor Va increases the catalytic efficiency of the enzyme (Rosing et al., 1980). This suggests a role for factor Va in the interaction between prothrombinase and substrate (prothrombin). The equilibrium binding studies presented here indicate that factor Va facilitates the binding of prothrombin to the phospholipid surface. Under the conditions of the experiments, of which the results are presented in Table I, factor Va decreases the dissociation constant of prothrombin-phospholipid (20% DOPS) complexes approximately 40-fold. We have to emphasize that we are dealing with apparent dissociation constants, dependent on the DOPS content of the vesicles. It appears from Figure 2 that at high factor Va concentrations, the amount of prothrombin bound to the vesicle decreases with increasing factor Va concentration. This strongly suggests that the additive effect of protein-protein interaction as an important factor in facilitating the binding of one of the components, i.e. prothromin, to the phospholipid surface is abolished by a competition with factor Va for the same binding sites. In accordance with this notion, there appears to be a 3-fold increase in the apparent dissociation constant of prothrombin-phospholipid complexes in the presence of factor Va when the DOPS content of the vesicles is lowered from 20% to 10% (Table I). A comparison of these results with the data obtained under identical conditions from factor Xa binding studies in the presence of factor Va reveals that: 1) the apparent dissociation constant of factor Xa-phospholipid complexes in the presence of factor Va is approximately 5-fold lower, 2) decreasing the DOPS content of the vesicles has less effect on the dissociation constant and 3) factor Xa is not competitively removed from the phospholipid by high factor Va concentrations (Figure 1). These findings can be qualitatively rationalized by assuming that the affinity of prothrombin towards factor Va is much less then the affinity of factor Xa towards factor Va. The dissociation constant of the latter complex in the absence of phospholipid has been reported to be about 4×10^{-9} M (Lindhout et al., 1982).

A decrease of several orders of magnitude of the apparent dissociation constant of prothrombin-phospholipid complexes by factor Va might be relevant for prothrombin activation by factor Xa. Kinetic studies of prothrombin activation have shown that factor Va greatly decreases the K_M for prothrombin when vesicles contain low percentages di-oleoyl-phosphatidylserine (van de Waart et al., 1983b). Another explanation for the additive effect of protein-

protein interaction as a dominant factor in the increase in affinity of prothrombin to a phospholipid surface has to be considered. It cannot be ruled out that acidic phospholipids are clustered around a factor Va molecule. If so, factor Va might modulate the phospholipid surface by providing a higher negatively charged phospholipid content at its vicinity as compared with the bulk-surface. To this end, equilibrium binding studies were performed utilizing the phospholipid-binding subunit of factor Va (factor Va LC).

We demonstrated that the dissociation constants of factor Xa-, prothrombin- and factor Va- phospholipid complexes increase with the concentration of factor Va LC. Because the phospholipid binding properties of factor Va LC are identical with those of factor Va, we conclude from these observations that modulation of the phospholipid surface by factor Va is not enhancing the interaction of phospholipid with prothrombinase proteins.

In addition, the results of this study also provide us with information about the structure-function relationship between factor Va subunits and the other protein components of the prothrombinase complex, factor Xa, as well as prothrombin. In contrast to the findings of Tracy & Mann (1983) utilizing platelets, we could not establish that the 80,000 dalton component of factor Va (factor Va LC) also serves as the binding site for interaction of factor Xa with phospholipid-bound factor Va. Our results confirm previously reported evidence that the Ca^{++}-mediated interaction between the two subunits of factor Va is required for binding of factor Va to factor Xa (Lindhout et al., 1982).

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CHAPTER VI

THE EFFECT OF FACTOR Va ON K_m FOR PROTHROMBIN IN THE PROTHROMBINASE REACTION AND ON THE ROTATIONAL MOTION OF PROTHROMBIN.

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The effect of factor Va on the kinetic parameters of prothrombin activation by factor Xa was studied at varying DOPS content of the membrane. The K_m for prothrombin decreased and the k_{cat} increased with increasing mole fractions of DOPS. Factor Va decreases the K_m at low mole fraction of DOPS. At 200 μM phosphlipid (2.5% DOPS) the K_m for prothrombin decreased from 3.5 μM in the absence to 0.1 μM in the presence of factor Va. At 50 μM phospholipid the K_m values were 1.5 and 0.1 μM respectively. Factor Va had a minor effect on the K_m when the phospholipid vesicles contained more than 20% DOPS. In the presence of factor Va and vesicles that contained 5 to 35% DOPS, the k_{cat} was found to be independent of the mole fraction DOPS. Between 1 and 5% DOPS a slight increase in k_{cat} was observed with increasing mole fraction DOPS. The interaction of FITC-labeled prothrombin with factor Va in the absence and the presence of phospholipid vesicles was studied by use of fluorescene anisotropy decay measurements. In all cases studied the anisotropy decay could be fitted with two correlation times: a very short one, characteristic for a rapid independent rotation of the label itself that is flexible bound to the prothrombin, and a longer one reflecting rotation of labeled prothrombin or prothrombin fragments. The longer correlation time lengthened when both phospholipid vesicles and factor Va were present, indicating a very specific protein-protein association on the vesicle surface. In the absence of phospholipid no interaction between the two proteins could be observed, indicating a rather weak binding interaction. Our results suggest

that when phospholipids are utilized with low affinities for factor Xa and prothrombin, factor Va-prothrombin interaction greatly enhances the affinity of the prothrombinase complex for its substrate prothrombin, and as a result a tighter binding interaction at the active site occurs.

INTRODUCTION

The phospholipid component of the prothrombinase complex serves as a surface on which factor Xa and prothrombin bind (see Stenflo & Suttie, 1977 for a review). Rosing et al. (1980) demonstrated that phospholipids lower the K_m for prothrombin. It was proposed that the phospholipid surface serves as a concentrating devise to produce a dense shell of locally high prothrombin and factor Xa concentrations; the so-called dense shell theory. The K_m for prothrombin would be a function of the density of the substrate at the surface (Nesheim et al., 1981)

Another explanation for the effect of phospholipids was given by Nelsestuen (1978): Due to the additive free energies of protein-protein interactions plus protein-phospholipid interactions, a tighter binding interaction occurs at the active site; the K_m for prothrombin would be a function of the prothrombin concentration in free solution (additive free energy model).

The protein cofactor component of the prothrombinase complex, i.e. factor Va, is known to increase the rate of thrombin formation by many orders of magnitude. Factor Va exerts its effect probably because of its interaction with both factor Xa and prothrombin (Rosing et al., 1980; Van de Waart et al., 1984). However, factor Va appears not or hardly to affect the Km for prothrombin, either in the absence or presence of phospholipids (Rosing et al., 1980). In terms of the dense shell model, this is easily to conceive because the prothrombin concentrations largely exceede the factor Va concentrations. Therefore, factor Va would not contribute to an increased prothrombin density at the surface.

If protein-protein interaction plus protein-phospholipid interactions cause a tighter binding interaction at the active site and thus would affect the kinetic parameters (Nelsestuen, 1978), the question arise why in the

kinetic studies of Rosing et al. (1980) the K_m for prothrombin was not found to differ significantly when factor Va was present or not. In these studies a membrane composition was chosen that bound prothrombin rather strongly (Nelsestuen & Broderius, 1977). Therefore it is interesting to study the effect of factor Va on the K_m for prothrombin on vesicles that weakly bind prothrombin, i.e. vesicles with low mole fractions of DOPS. To this end we varied the DOPS mole fractions from 0.01 to 0.35 in our studies.

Rotational motion of biological macromolecules can be conveniently monitored via anisotropy decay measurements of an appropriate fluorescent marker molecule (J. Yguarabide, 1972; Ph. Wahl, 1975). The method not only provides information on local motion of either the probe itself or a small peptide fragment attached to it, but also on the interactions between enzymes and asseccory components in the catalysis of a specific biological reaction. Such examples have been reported recently (Visser & Lee, 1982; Visser et al., 1983).

In order to gain insight into the effect of factor Va on the vectorial organization of prothrombin on the phospholipid surface, we monitored the rotational motion of FITC-prothrombin via anisotropy decay measurements.

MATERIALS AND METHODS

Prothrombin, factor Xa and factor Va were prepared and quantitated as described before (Van de Waart et al., 1983b). Rates of thrombin formation were measured essentially by the method of Rosing et al. (1980). Thrombin formation at varying prothrombin concentrations was measured at 37 ^{o}C in reaction mixtures containing 50 mM Tris, 100 mM NaCl, ovalbumin (0.5 mg/ml), pH 7.5, fixed amounts of factor Xa, phospholipid and calciumchloride in the absence or presence of factor Va. The reaction was initiated by the addition of prothrombin. The kinetic parameters were determined from Lineweaver-Burk plots and evaluated as described by Rosing et al (1980). Phospholipid vesicles of varying mole fractions 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) were prepared as previously described (Lindhout et al., 1982).

Prothrombin was labeled with fluorescein isothiocyanate (FITC) isomer 1 (Nordic) according to Fass & Mann (1973). The molar ratio of FITC to protein ranged from 1.0 to 2.0. The labeling was found to be random, i.e. after activation of prothrombin by factor Xa-factor Va-phospholipid complex, all fragments normally found in native prothrombin could be detected by means of fluorescence upon SDS-polyacrylamide gel electrophoresis. Labeled prothrombin could not be distinguished from native prothrombin by kinetic analysis of the activation process of prothrombin.

The anisotropy decay measurements were carried out as previously described (Visser & van Hoek, 1979; Visser, 1982; van Hoek et al., 1983). We used the mode-locked 496 nm line of an Argon ion laser and an electro-optical modulator for scaling down the pulse repitition rate of the mode-locked laser pulses (van Hoek & Visser, 1981). Fluorescence was selected via a Balzars K51 band-pass filter. The photon-counting data were stored in two halves of the memory of a multichannel analyzer, one registrating the emission component parallel to the polarization of the excitation [$I_{//}(t)$], the other the perpendicularly polarized emission [$I_{\perp}(t)$]. Ten cycles of 10 seconds for each component were usually sufficient to obtain significant data. Background data resulting from a similar experiment, but in the absence of the implemented fluorophore, were directly substracted from fluorescence data. The data were transferred to a DEC-10 computer for further evaluation. From the separate components two experimental quantities were constructed. The first was the total fluorescence $S(t) = I_{//}(t) + 2I_{\perp}(t)$, from which the fluorescence lifetimes were obtained. The second one was the time-dependent anisotropy $r(t) = [I_{//}(t) - I_{\perp}(t)]/S(t)$, from which rotational corralation times were evaluated. Both sets of parameters were obtained after an iterative nonlinear least square search procedure. Details have been given elsewhere (Visser & van Hoek, 1979; Visser, 1982; van Hoek et al., 1983). Time-resolved fluorescence experiments were all carried out at 20 ^{o}C in a buffer containing 50 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$ and ovalbumin (0.5 mg/ml), pH 7.5.

RESULTS AND DISCUSSION

Determination of Optimal Calcium Concentrations

The binding of factor Xa, prothrombin and factor Va to phospholipid and therefore of the rate of thrombin formation, depends on the calcium concentration. Optimal calcium concentrations were determined from calcium titrations under various conditions of prothrombin activation. The phospholipid concentration, membrane composition and prothrombin concentration were varied either in the absence or presence of factor Va.

Figure 1 shows typical plots of normalized rates of thrombin formation versus the Ca^{++} concentration. In the absence of factor Va, the maximal rate of thrombin formation was obtained at 10 mM Ca^{++} for vesicles containing 2.5 or 25% DOPS (Figure 1A). In the presence of factor Va lower Ca^{++} concentra-

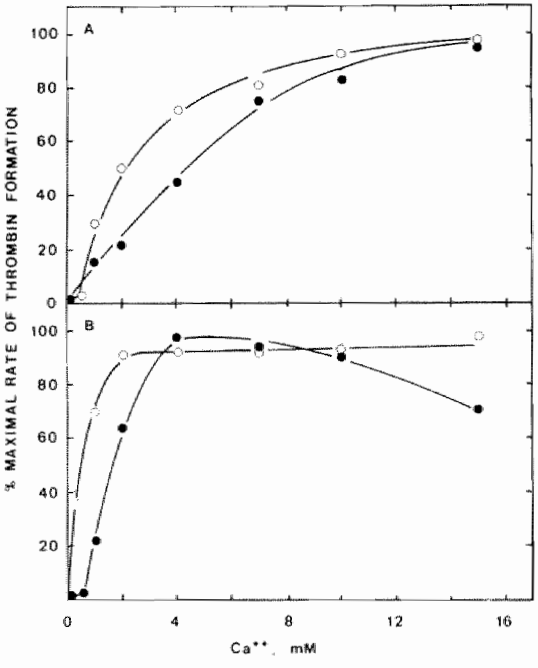

FIGURE 1: The Ca^{++}-dependence of thrombin formation in the absence and presence of factor Va. Panel A, prothrombin (0.2 μM) was incubated at 37 °C with factor Xa (5.0 nM) in buffer containing 50 mM Tris, 100 mM NaCl, 0.5 mg/ml of ovalbumin, pH 7.5 and varying amounts of calcium in the presence of 25 μM phospholipid. Panel B, prothrombin (30 nM) was incubated with factor Xa (5.0 pM), factor Va (1.0 nM) and phospholipid (25 μM). Further experimental details are as described above. The phospholipids were DOPS/DOPC [(2.5/97.5), (●)] or DOPS/DOPC [(25/75), (○)].

tions are required, 2 and 4 mM Ca^{++} in case of vesicles containing 25% and 2.5% DOPS, respectively (Figure 1B). It is interesting to see that in case of vesicles containing 2.5% DOPS, the rate of thrombin formation decreased with increase of Ca^{++} concentration when factor Va is present. A potential explanation for this effect might be that calcium ions compete with factor Va for phospholipid binding sites (Van de Waart et al., 1983). At higher phospholipid concentrations this effect was not observed. We further found that the normalized rates of thrombin formation did not change with the prothrombin concentration.

From these data we concluded that 10 mM Ca^{++} was a suitable concentration to be used in our kinetic studies.

The Influence of Factor Va and the Mole Fraction DOPS of the Vesicles on the Kinetic Parameters of Prothrombin Activation

Lineweaver-Burk plots were constructed from data obtained from prothrombin activation experiments utilizing artificially prepared membranes containing various mole fractions DOPS, factor Xa, calcium ions and either in the absence or presence of factor Va. The reciprocal plots of rates of thrombin formation versus prothrombin concentrations yielded straight lines (data not shown).

Figure 2A shows the maximal rate of thrombin formation as a function of the DOPS content of the vesicles in the absence of factor Va. It is demonstrated that the k_{cat} values increase with the increase in DOPS content. This effect is consistent with the observation that the affinity of factor Xa for membranes increases with DOPS content (Nelsestuen & Broderius, 1977; van Dieijen et al., 1981). Therefore, at a fixed factor Xa concentration the amount of effective enzyme, i.e. phospholipid-bound factor Xa, will increase with DOPS content. This also provides an explanation for the higher k_{cat} values at higher phospholipid concentrations.

As known, the k_{cat} increases greatly when factor Va is present (Rosing et al., 1980). It is apparent that in the presence of factor Va, the k_{cat} did not differ significantly with the DOPS content (Figure 2B). A slight increase was observed between 1 and 5% DOPS. Evidently, because of the coordinate binding interaction between factor Va and factor Xa at the phospholipid surface, factor Xa is quantitatively bound to the surface independent of the

DOPS content and phospholipid concentrations used (Lindhout et al., 1982).

Figure 3 depicts the K_m values for prothrombin as a function of DOPS content of the membrane and factor Va. In the absence of factor Va the K_m increased greatly with decreasing DOPS content, e.g.: K_m = 0.6 μM (20% DOPS, 200 μM), K_m = 3.5 μM (2.5% DOPS, 200 μM) and K_m = 0.3 μM (20% DOPS, 50 μM), K_m = 1.5 μM (2.5% DOPS, 50 μM). It is apparent that the increase of K_m below 15% DOPS reflects the decreased binding affinity of prothrombin for the membrane. According to the dense shell model, the prothrombin concentration has to be increased in order to maintain the required substrate density at the surface. The additive free energy model predicts the same effect on basis of decreased free energies of prothrombin-phospholipid interaction. The increase in K_m with increasing phospholipid concentration might be due to either a reduction of the substrate density as a result of the increased number of prothrombin binding sites (dense shell model) or caused by the

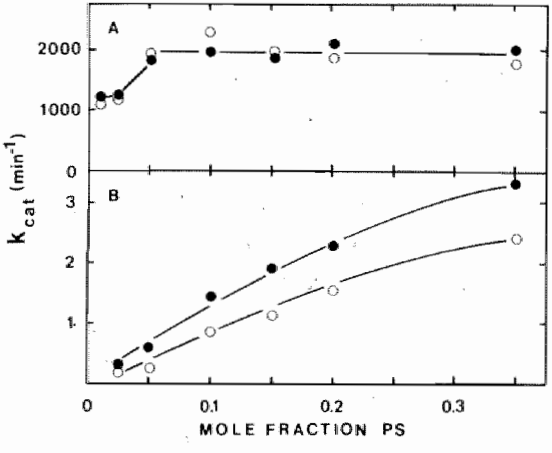

FIGURE 2: The effect of factor Va on k_{cat} of prothrombin activation. Panel A, thrombin formation at varying prothrombin concentrations was measured at 37 ^{o}C in a reaction mixture containing factor Xa (2.5 pM), factor Va (2 nM), phospholipid [50 μM, (○); 200 μM, (●)] and 10 mM $CaCl_2$, 50 mM Tris, 100 mM NaCl, 0.5 mg/ml ovalbumin, pH 7.5. Panel B, prothrombin activation was carried out in a mixture containing factor Xa (5 nM), phospholipid [50 μM, (○); 200 μM, (●)], and varying prothrombin concentrations. Further experimental details as described above. The k_{cat} values were calculated from Lineweaver-Burk plots.

competitive removal of substrate from the active center (additive free energy model).

Attempts to determine the kinetic parameters in the absence of factor Va utilizing vesicles containing 1% DOPS were hampered by the fact that curved Lineweaver-Burk plots were obtained. Apparently, the rate of thrombin formation in free solution at high prothrombin concentrations exceeded the rate of the phospholipid surface-catalyzed formation of thrombin. Therefore, those data were not included in Figure 3.

Factor Va had a striking effect on the K_m for prothrombin when vesicles were used that contained less then 20% DOPS. By increasing the phospholipid concentration, this effect became even more pronounced. Factor Va decreased the K_m for prothrombin from 3.5 μM to 0.1 μM when phospholipids (200 μM) were used that contained 2.5% DOPS. In the dense shell model this effect cannot be explained; unless we have to assume that factor Va modulates the phospholipid in a way that may promote the binding of prothrombin (for example clustering or inducing transbilayer movement of negatively charged phospholipids). Evidence against that was provided in a recent report on the binding interaction between factor Va, factor Va LC and prothrombin (van de Waart et al., 1984). However, the effect of factor Va on K_m is well explained within the

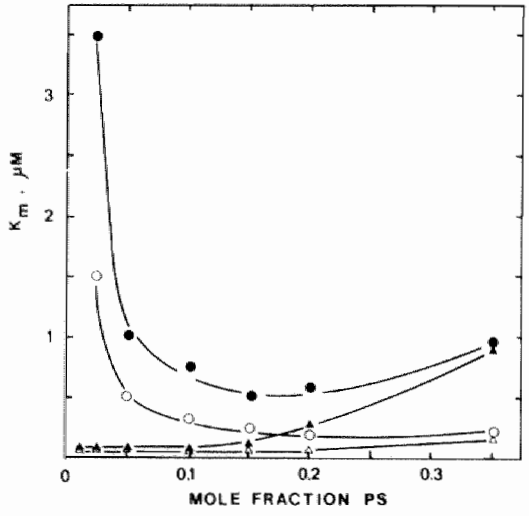

FIGURE 3: The effect of factor Va on K_m for prothrombin. The prothrombin activation mixtures were as described in figure 2. (●), no factor Va present, 200 μM phospholipid; (○), no factor Va present, 50 μM phospholipid; (▲), 2 nM of factor Va, 200 μM phospholipid; (△), 2 nM of factor Va, 50 μM phospholipid.

additive free energy model. If we assume that K_m is related to a binding constant and by virtue of the binding interactions between Factor Va, factor Xa and prothrombin, the additional protein-protein interactions as provided by factor Va would result in a stronger overall interaction at the active site and a lower K_m.

It is apparent that the K_m for prothrombin did not differ significantly, whether or not factor Va is present, in case the membranes contained >20% DOPS. This suggests that increasing prothrombin-phospholipid binding affinity with increase in DOPS content of the membrane, masks the by factor Va induced additive free energy of protein-protein interaction as an important contribution to the tighter binding at the active site. Under these conditions a large number of relatively high affinity phospholipid binding sites for prothrombin, competitively removes prothrombin from the active site, i.e. phospholipid-bound factor Xa-factor Va, as indicated by the observation that the prothrombin concentration had to be increased in order to obtain half-saturation of the enzyme with substrate.

Pusey & Nelsestuen (1983) demonstrated that a judicious selection of membrane composition to which prothrombin was bound very weakly, allowed nearly optimal kinetics when the vesicles outnumbered the prothrombin molecules. This observation also precludes the formation of a dense shell of protein at the membrane as an important mechanism of membrane function. Taking the results together, it is clear that the prothrombinase complex can be viewed as a dissociable three component enzyme (factor Xa-factor Va-phospholipid) that forms a Michaelis-Menten complex with prothrombin from solution.

The Effect of Factor Va on the Rotational Motion of Prothrombin on Phospholipid Vesicles

The fluorescence decay curves of FITC-prothrombin could not be fitted with one exponential term. The minimum decay model that describes the decay was the sum of two exponentional terms, i.e. a short lifetime component in the order of 0.4 - 1.2 ns and a longer one between 3.5 and 3.9 ns. A typical example is shown in Figure 4A. The value of the average lifetime was invariant under the conditions used (Table I). The exact nature of the non-exponentiality has not been further explored, but nonexponentional fluores-

cence decay is a quite common phenomenon in biological samples.

The fluorescence anisotropy decay measurements, on the other hand, revealed changes which could be related to changes in rotational behaviour of prothrombin. The time-dependent anisotropy of the same sample of Figure 4A has been given in Figure 4B. The analysis yielded in all cases investigated two relaxation times: a short one of about (1.5 ns) and a longer one between 30 and 100 ns, the value of which depended on the conditions used. The most significant decay patterns are plotted together in Figure 5. All the correlation times have been collected in Table I.

The short relaxation time of 1.5 ns must arise from a rapid probe motion, probably around its point of attachment to the protein. Superimposed on the

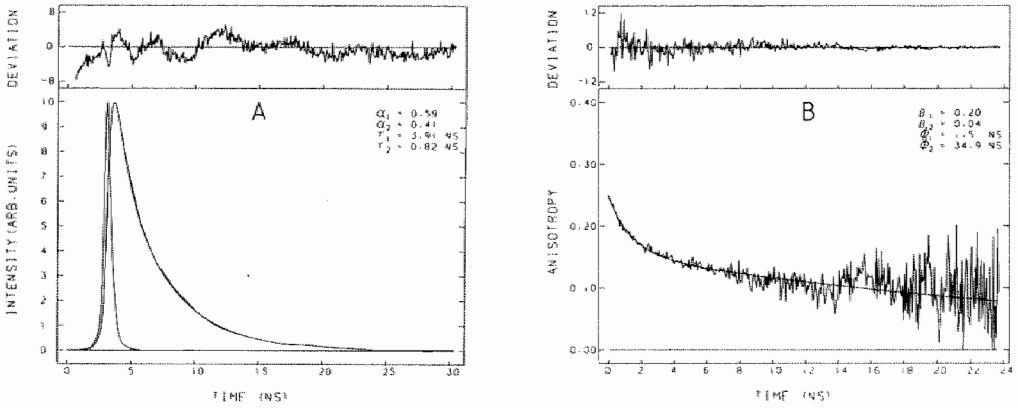

Figure 4: Fluorescence (A) and Anisotropy (B) Decay of FITC-prothrombin (5nM) in the Presence of Factor Va. A: Shown are the laser pulse profile, the experimental fluorescence S(t) and calculated fluorescence $D_c(t)$ composed of the convolution product of the laser pulse with a double exponential decay function $\alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$. The optimized parameter values α_i, τ_i are given in the figure. The deviation function as function of time (top panel) is given by $[S_c(t) - S(t)]/\sqrt{S(t)}$. B: Shown are the experimental anisotropy r(t) (noisy curve) and the fit to a double exponential function $r_c(t) = \beta_1 \exp(-t/\varphi_1) + \beta_2 \exp(-t/\varphi_2)$. The optimized parameter values β_i,φ_i are given in the figure. The time-dependent deviation function is given by $[r_c(t)-r(t)]/\sqrt{V(t)}$, V(t) is the variance of r(t). Analysis started at the time channel corresponding to the maximum of the laser pulse.

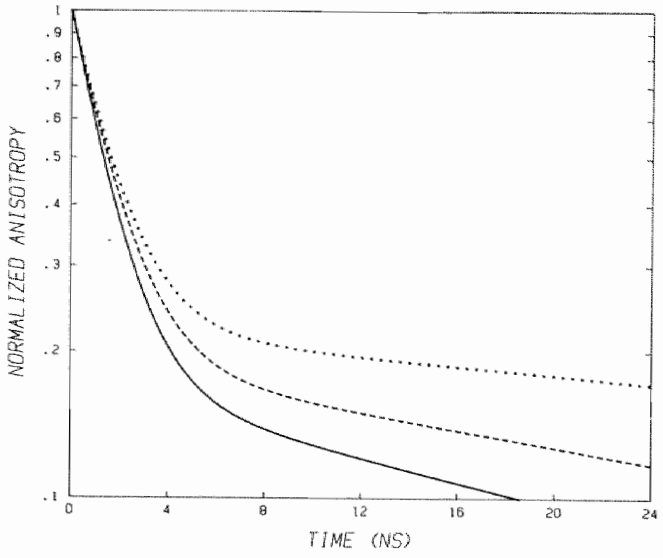

Figure 5: Normalized anisotropy Decay Curves of FITC-prothrombin under Different Conditions. (———) 5 nM FITC-prothrombin, 1 μM Va; (– – – –) 5 nM FITC-prothrombin, 50 μM phospholipid; (·····) 2 nM FITC-prothrombin, 0.1 μM Va and 50 μM phospholipid. Parameters have been listed in Table I.

component of rapid rotation is a slower component, which must be ascribed to rotational depolarization of a larger protein fragment. The significant contribution of the short component in the anisotropy decay impedes an unambiguous interpretation of the results. However, we can draw important, qualitative conclusions regarding interactions involving prothrombin. In a binary mixture of prothrombin and factor Va we did not observe a lenthening of the long relaxation time. This indicates no formation of a protein-protein complex during the time scale of the experiments even at large excess of Va. Neither the steady state anisotropy <r> nor the long correlation time showed titration behaviour (Table I), they were invariant upon changes in solution conditons. If a complex between factor Va and prothrombin is formed the interaction must be weak with a dissociation constant higher than 10 μM (Table I, entry 2). In the presence of phospholipid and in the absence of Va we observed a slightly longer correlation time indicating a more restricted motion of the vesicle-bound prothrombin. The effect is, however, small and the protein is still able to perform a large amplitude motion on the vesicle surface. The addition of factor Va, thus constituting a ternary system,

induced a much larger motional restriction in the prothrombin fragment because of the significantly longer correlation time, φ_2, determined in this case. The motional restriction of prothrombin can be explained by assuming that factor Va and prothrombin associate on the vesicle surface. In contrast to the binding of prothrombin to phospholipid both in the absence and presence of factor Va the steady state anisotropy <r> and the long correlation time does not show titration behaviour and again this has to be ascribed to the large amplitude motion of prothrombin even in the presence of phospholipid and factor Va. In addition to a tighter binding interaction at the active site of prothrombinase, such a vectorial organization on the surface of the membrane bilayer might be significant for a more effective encounter rate between prothrombinase and prothrombin and thus the effective catalytic formation of thrombin. More specificly labeled prothrombin or factor Va, e.g. with the probe more tightly bound to well-defined protein fragments, will be usefull to confirm this hypothesis and may yield information about the structure function relation concerning the interaction sites.

Table 1: Fluorescence and Anisotropy Decay Characteristics of FITC-Prothrombin

Sample	Fluorescence lifetimes					correlation times					anisotropy
	α_1	τ_1(ns)	α_2	τ_2(ns)	$\langle\tau\rangle$(ns)	β_1	ϕ_1(ns)	β_2	ϕ_1(ns)	$\langle\phi\rangle$(ns)	$\langle r\rangle$
F-PT*, 800 nM	0.53	0.4	0.47	3.5	3.5	0.19	1.7	0.06	45	2.1	0.18
F-PT,2-800 nM[c] Va, 1 μM	0.46 ±0.08	0.7 ±0.3	0.54 ±0.08	3.8 ±0.1	3.5	0.20 ±0.01	1.7 ±0.3	0.05 ±0.01	50 ±15	2.1 ±0.2	0.18
F-PT,5-100 nM[d] PL, 50 μM	0.40 ±0.03	1.0 ±0.3	0.60 ±0.02	3.9 ±0.1	3.5	0.19 ±0.01	1.7 ±0.01	0.05 ±0.01	48 ±2	2.1 ±0.2	0.17
F-PT,2-100 nM[e] PL, 50 μM Va, 0.1 μM	0.35 ±0.04	1.14 ±0.05	0.65 ±0.04	3.92 ±0.01	3.5	0.19 ±0.01	1.6 ±0.1	0.06 ±0.01	85 ±10	2.2 ±0.1	0.18

* F-PT: FITC-prothrombin; PT: prothrombin; Va: blood clotting factor Va; PL: phospholipid vesicles containing 20% DOPS and 80% DOPC.

a) $\langle\tau\rangle = (\alpha_1\tau_1^2 + \alpha_2\tau_2^2)/(\alpha_1\tau_1 + \alpha_2\tau_2)$

b) $\langle\phi\rangle = (\beta_1 + \beta_2)\,\phi_1\phi_2\,/(\beta_2\phi_1 + \beta_1\phi_2)$

c) Average of 6 experiments: 2,4,10,50,200 and 800 nM F-PT

d) Average of 3 experiments: 5,20 and 100 nM F-PT

e) Average of 3 experiments: 2,20 and 100 nM F-PT

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CHAPTER VII

FUNCTIONAL PROPERTIES OF FACTOR VA SUBUNITS AFTER PROTEOLYTIC ALTERATIONS BY ACTIVATED PROTEIN C

Piet van de Waart, Harry Bruls, H.Coenraad Hemker and Theo Lindhout

The two-subunit structure of the factor Va molecule is essential to its function in the prothrombinase complex. In the presence of phospholipids, the cleavage of the light chain of bovine factor Va by activated protein C proceeded at the same rate as the cleavage of the heavy chain. The limited proteolysis of factor Va is accompanied by a parallel loss of factor Va activity. Evidence that loss of activity was solely the result of the cleavage of the heavy chain, was obtained from reconstitution experiments utilizing cleaved and intact chains. The pseudo first-order rate constant of factor Va inactivation by activated protein C was found to be dependent on the amount of phospholipid-bound activated protein C and not on the amount of phospholipid-bound factor Va. However, phospholipids enhance the rate of proteolysis of the phospholipid-binding subunit, i.e. the light chain, and not the cleavage of the heavy chain. Cleavage of the heavy chain and as a consequence the inactivation of factor Va by activated protein C is mediated by the light chain. After cleavage of the light chain, the "two-subunit" stucture, as well as the phospholipid-binding properties of factor Va were found to be conserved.

INTRODUCTION

Activated protein C, which is derived by limited proteolysis of the zymogen by thrombin, trypsin or factor X-converting protein from Russell's viper venom (Kisiel, et al., 1976; Kisiel, et al., 1977; Walker et al.,1979)

the inactivation of factor Va and factor VIII:C by activated protein C (Seegers et al., 1978; Walker et al., 1979; Vehar & Davie, 1980). Current knowledge about the molecular events that lead to the inactivation of factor Va by activated protein C has emerged from studies on factor Va degradation, utilizing SDS-polyacrylamide gel electrophoresis (Walker et al., 1979; Tracy et al., 1983; Suzuki et al., 1983). It was inferred that proteolysis of the 94,000 dalton subunit of factor Va (factor Va HC) results in the inactivation of factor Va. Whether proteolysis of the 80,000 dalton subunit (factor Va LC) affects the functional properties of factor Va is not known.

Phospholipids greatly enhance the rate of factor Va inactivation by activated protein C (Kisiel et al., 1977; Walker et al., 1979; Suzuki et al., 1983). Activated protein C and factor Va bind to phospholipid with dissociation constants of $\sim 10^{-5}$ M (Nelsestuen et al., 1978) and $\sim 10^{-8}$ M (Van de Waart et al., 1983) respectively. However, it is an open question whether both proteins have to bind to phospholipid in order to obtain an efficient inactivation reaction (Stenflo, 1976). Therefore, in view of the observations that the factor Va HC is bound to phospholipid by means of factor Va LC (Van de Waart et al., 1983) and phospholipids stimulate the cleavage of factor Va HC if factor Va LC is present (Walker et al., 1979; Suzuki et al., 1983) the question has to be adressed as to the organization of the factor Va molecule on the phospholipid surface, when factor Va and especially factor Va LC is processed by activated protein C.

This study of the structural alterations in the subunits of factor Va as induced by activated protein C was undertaken to obtain better insight into the consequences as to the functional properties of factor Va and the inactivation process of factor Va. A preliminary account of this work has been published (Bruls et al., 1983)

MATERIALS AND METHODS

Russell's viper venom, ovalbumin and dioleoylphosphatidylcholine were from Sigma Chemical Co. The chromogenic substrate D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride (S2238) was partially donated by AB Kabi Diagnostica.

Preparation of Proteins

Protein C was isolated from bovine plasma by a modification (Walker et al., 1979) of the method according to Stenflo (1976). Factor X-activator from Russell's viper venom (RVV-X) was purified as described by Schiffman et al. (1969). Protein C was activated with RVV-X and purified as reported by Walker et al. (1979). Activated protein C concentrations were calculated from the absorbance at 280 nm using $E_{280}^{1\%} = 13.7$ (Kisiel et al., 1976). Bovine factor V and factor Va were prepared by a modification (Lindhout et al., 1982) of the method of Esmon (1979). Factor Va subunits and all other proteins used in this study were purified and quantitated as described previously (Van de Waart et al., 1983).

Factor Va Assay

A sample (10-100 l) containing factor Va was incubated with factor Xa (1.3×10^{-11} M), phospholipid (1.0×10^{-5} M), and $CaCl_2$ (1.0×10^{-2} M) in 50 mM Tris, 100 mM NaCl, 0.5 mg ovalbumin/ml in a final volume of 0.9 ml for 5 min at 37 °C in a plastic cuvette. The reaction was initiated by the addition of 0.1 ml of prothrombin (2.0×10^{-6} M). After 2 min, 1.0 ml buffer containing 50 mM Tris, 100 mM NaCl, 0.5 mg of ovalbumin, 20 mM EDTA and 0.47 mol S2238 was added to the reaction mixture. The amount of thrombin formed was calculated from the absorbance change as monitored with an Aminco DW-2 spectrophotometer operating in the dual wave-length mode (λ_s = 405 nm and λ_r = 500 nm), thermostated at 37 °C. A factor Va preparation of which the molar concentration was determined as described previously (Van de Waart et al., 1983) was used for the construction of standard curves.

Iodination of Factor Va Subunits

Factor V was iodinated using Bolton-Hunter reagent (Bolton & Hunter, 1973). ^{125}I-Factor V was activated with thrombin and the ^{125}I-labelled factor Va subunits were isolated as described previously (Suzuki et al., 1982).

Proteolysis of Factor Va Subunits by Activated Protein C

Factor Va LC (5 mg, 0.5 mg/ml) in 20 mM Tris, 50 mM NaCl, 3 mM $CaCl_2$, pH 7.5 was incubated with activated protein C (10 nM) in the presence of 20 μM phospholipid for 10 min at 37 ^{o}C. Upon complete conversion of factor Va LC, activated protein C was quantitatively removed from the mixture by chromatography on a SP-Sephadex column (0.9x10 cm) and washing the column with the above buffer. Factor Va LC proteolysis products, i.e. fragments with Mr 51,000 and Mr 32,000 were eluted from the column with 20 mM Tris, 200 mM NaCl, pH 7.5.

Factor Va HC (7 mg, 0.7 mg/ml) in 20 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.5 was incubated with activated protein C (0.5 μM) for 90 min at 37 ^{o}C. After complete conversion of factor Va HC, the reaction mixture was applied to a QAE-Sephadex column (0.9x10 cm) equilibrated in the above buffer omitting Ca^{++}. The factor Va HC proteolysis products, i.e. fragments with M_r = 72,000 and M_r=24,000, were eluted from the column with 20 mM Tris, 350 mM NaCl, pH 7.5.

HPLC of Factor Va Subunits

Size-exclusion high-performance liquid chromatography was carried out on Spherogel TSK 3000 SW (7.5x600 mm) in 20 mM Tris, 700 mM NaCl, pH 7.5 at a flow rate of 1.0 ml/min. The instrument was a Beckmann Model 500 high-performance liquid chromatograph equipped with a Beckmann Model 160 Absorbance Detector. A 280-nm filter was used to detect the protein peaks. Samples (100 μl) were injected after a 30-min period of incubation at 37 ^{o}C in the column buffer.

Protein-binding Measurements

Binding of protein to phospholipid was measured as previously described (Van de Waart et al., 1983). Briefly, mixtures of large-volume vesicles and protein were incubated for 10 min at ambient temperature. Prior to and after centrifugation for 30 min at 30,000xg, aliquots were withdrawn and assayed for protein for determination of total protein concentration and the concentration of unbound protein, respectively.

Phospholipid Preparations

Large-volume and sonicated phospholipid vesicles containing 80% (w/w) dioleoylphosphatidylcholine and 20% (w/w) dioleoylphosphatidylserine were prepared as described previously (Van de Waart et al., 1983).

RESULTS

Degradation of Bovine Factor Va by Activated Bovine Protein C

Thrombin-activated factor V (unfractionated factor Va) consists of four polypeptides, e.g. factor Va LC (M_r=80,000), factor Va HC (M_r=94,000), a 150-kDa fragment which is very rich in carbohydrate and a 65-kDa fragment. Factor Va LC is associated via calcium ions with factor Va HC and together constitute active factor Va (Lindhout et al., 1982; Esmon, 1979). Identical activation fragments have been reported for thrombin-activated human factor V (Suzuki et al., 1982).

Unfractionated factor Va was incubated with activated protein C in the presence of phospholipids and the decrease in factor Va activity was compared with proteolytic events as monitored by SDS-polyacrylamide gel electrophoresis (Figure 1). Factor Va activity decreased in parallel with the degradation of both factor Va HC and factor Va LC, giving rise to fragments with M_r=72,000 - 24,000.

The 150-kDa and 65-kDa fragments present in the unfractionated factor Va preparation are not visualized by staining with Coomassie Blue. As monitored by SDS-polyacrylamide gel electrophoresis and visualized by staining with periodic acid-Schiff's reagent, we found that these fragments are not degraded by activated protein C (data not shown).

Isolated factor Va LC and factor Va HC were degraded by activated protein C to determine the origin of the degradation products. In accordance with the recently reported degradation pattern of human factor Va (Suzuki et al., 1983) we found that the fragments with M_r=51,000 and M_r=32,000 emerge from factor Va LC and factor Va HC is converted into fragments with M_r=72,000 and M_r=24,000. A further degradation of the 72-kDa fragment gave rise to a 47-kDa and 30-kDa fragment.

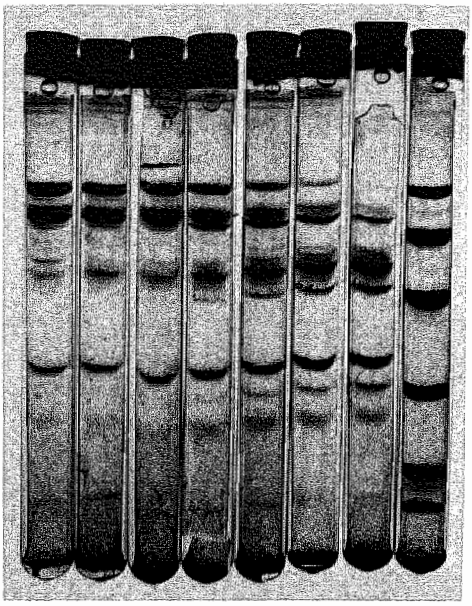

FIGURE 1: Degradation of thrombin-activated factor V by activated protein C. Factor Va (1.0 μM) in 50 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.5, phospholipid (100 μM) and activated protein C (20 nM) were incubated at 37 °C. At intervals, aliquots were removed for factor Va assay and 10% SDS-polyacrylamide gel electrophoresis. The respective incubation times (min) and factor Va activities (%, in parentheses) were: A, 0 (100); B, 0.5 (95); C, 1 (90); D, 4 (45); E, 8 (24); F, 15 (9); J, 30 (0). Gel H, molecular weight standards (phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400.

Effect of Phospholipids on the Rate of Proteolysis of Factor Va and its Subunits

The rate of inactivation of factor Va by activated protein C is stimulated by negatively charged phospholipid (Walker et al., 1979; Tracy et al., 1983; Suzuki et al., 1983). However, the kinetics of factor Va inactivation are poorly understood.

As a first attempt to investigate this problem, we tried to correlate the apparent first-order rate constant of inactivation, k, with concentrations phospholipid-bound factor Va and/or phospholipid-bound activated protein C.

Figure 2 shows the effect of phospholipid concentration on the apparent first-order rate constant. It is demonstrated that the rate constant is proportional with the phospholipid concentration. The amount of phospholipid-bound activated protein C also increased proportionally with the phospholipid concentration. However, at 30 μM phospholipid nearly 100% of total added factor Va was bound. Apparently, under conditions of first-order kinetics, the rate constant, k, is independent of the amount of unbound factor Va and the substrate density at the phospholipid surface, but depends on the amount of phospholipid-bound activated protein C.

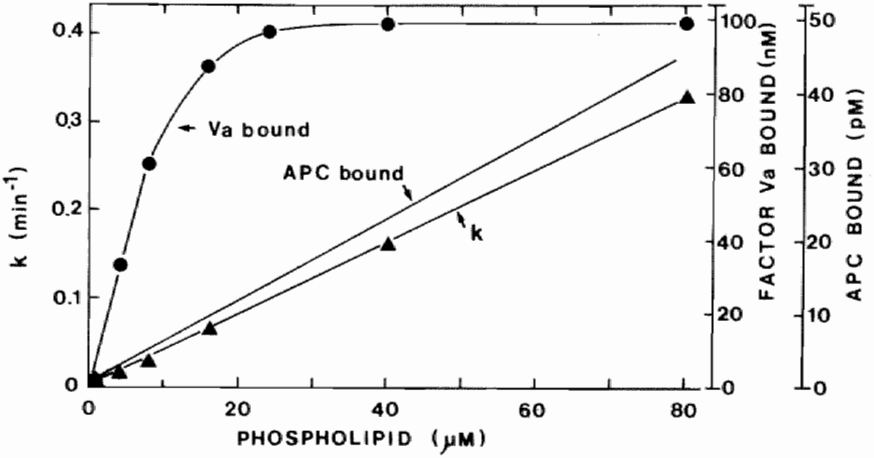

FIGURE 2: Effect of phospholipid concentration on the pseudo first-order rate constant of factor Va inactivation by activated protein C (APC). Factor Va (0.1 μM) in 50 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.5 was incubated with activated protein C (2 nM) in the presence of varying amounts of phospholipid. At intervals, aliquots were removed and assayed for factor Va activity. The rate constant, k (▲), was determined from the slope of a plot of log factor Va concentration versus time. The concentration of phospholipid-bound factor Va (●) was determined from identical reaction mixtures but omitting activated protein C as described in Materials and Methods. The concentrations of phospholipid-bound activated protein C (———) were calculated from published binding parameters (Nelsestuen et al. 1978)

However, inferrences as to whether phospholipid-bound factor Va or unbound factor Va is the preferred substrate cannot be drawn.

To this end, we investigated the effect of phospholipids on the rate of proteolysis of the non-phospholipid-binding (factor Va HC) and phospholipid-binding subunit (factor Va LC), by activated protein C. The time course of cleavage of ^{125}I-factor Va LC and ^{125}I-factor Va HC by activated protein C in the absence and presence of phospholipids was monitored by measuring the radioactivity in respective regions of SDS-polyacrylamide gels corresponding

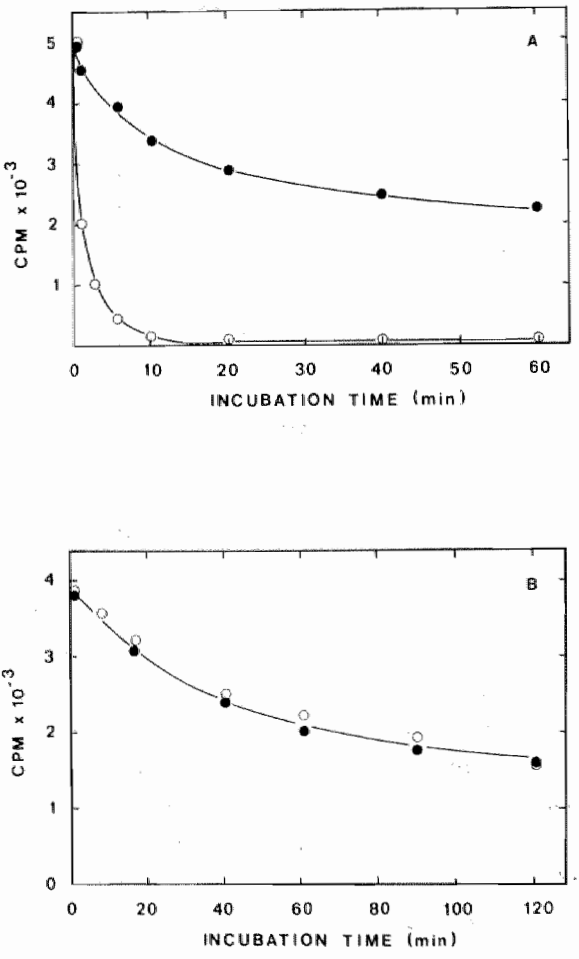

FIGURE 3: Effect of phospholipid on the rate of proteolysis of factor Va HC and factor Va LC by activated protein C. Panel A, ^{125}I-factor Va LC (0.3 mg/ml, 8x10^4 cpm/mg) in 20 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.5 was incubated with activated protein C (45 nM) in the presence of 50 μM phospholipid (O) or in the absence of phospholipid (●). At intervals, aliquots were removed and electrophoresed on 10% SDS-polyacrylamide gels. The gels were sliced in 2 mm sections and counted for radioactivity. Plotted is the radioactivity corresponding to ^{125}I-factor Va LC versus incubation time. Panel B, ^{125}I-factor Va HC (0.03 mg/ml, 4 x 10^6 cpm/mg) was incubated with activated protein C (1 μM) in the presence (O) or absence (●) of phospholipid. Further experimental details as described in Figure 3, panel A.

to the position of factor Va HC and factor Va LC. Figure 3 shows that the cleavage of factor Va LC is stimulated by phospholipid, while the rate of conversion of the subunit that does not bind to phospholipid (factor Va HC) is virtually unaffected by phospholipid. It is apparent that proteolysis by activated protein C is most effective when the substrate and activated protein C are bound to phospholipid.

Phospholipid-binding Properties of Proteolytic Altered Factor Va LC

Binding of factor Va to phospholipid is essential to the rate of inactivation of factor Va by activated protein C as it is essential to the function of factor Va in prothrombin activation. Since factor Va binding to phospholipid is mediated through factor Va LC (Van de Waart et al., 1983) it is of interest to investigate whether the phospholipid-binding properties of factor Va LC are altered after factor Va LC is degraded by activated protein C.

To this end, we performed a qualitative analysis of the phospholipid-binding properties of the factor Va LC degradation products. Unfractionated degraded factor Va LC was incubated with various amounts of large-volume vesicles. After centrifugation, the supernatants were analyzed by SDS-polyacrylamide gel electrophoresis. The amounts of 51-kDa and 32-kDa fragments

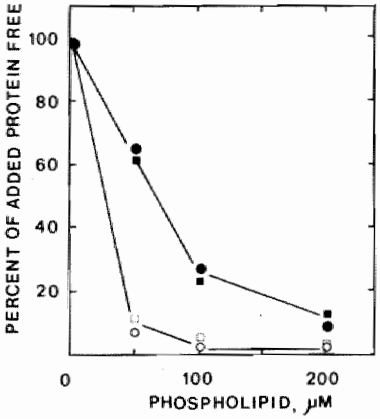

FIGURE 4: Phospholipid-binding properties of factor Va LC degradation products. A mixture of the 51-kDa and 32-kDa fragments (2 μM) obtained after degradation of factor Va LC by activated protein C was incubated with varying amounts of large-volume vesicles in 50 mM Tris, 100 mM NaCl, pH 7.5 in the presence of 2 mM EDTA (open symbols) or 5 mM $CaCl_2$ (closed symbols). The amounts of unbound protein as a percentage of total added protein are plotted versus phospholipid concentration. (○) and (●), 32-kDa fragment; (□) and (■), 51-kDa fragment. For further experimental details, see the text and Materials and Methods.

were quantitated by densitometric scanning and plotted versus the phospholipid concentration (Figure 4). It was demonstrated that both fragments bound equally well to phospholipid. The binding of both fragments could be reduced by adding calcium to the reaction mixture. We previously reported a similar effect of Ca^{++} for factor Va LC-phospholipid interaction (Van de Waart et al., 1983).

Factor Va HC-binding Properties of Proteolytic Altered Factor Va LC

The divalent cation-mediated interaction between factor Va HC and factor Va LC is essential to the activity of factor Va (Lindhout et al., 1982; Esmon, 1979). Figure 5 shows that factor Va activity, expressed as functional molar concentration (Van de Waart et al., 1983) could be restored by incubation of equimolar amounts of factor Va LC and factor Va HC in the presence of Mn^{++} ions. Titration of a fixed amount of factor Va HC with unfractionated factor Va LC degradation products, i.e. an equimolar mixture of 51-kDa and 32-kDa fragments, in the presence of Mn^{++}, also resulted in the almost complete restoration of active factor Va (Figure 5).

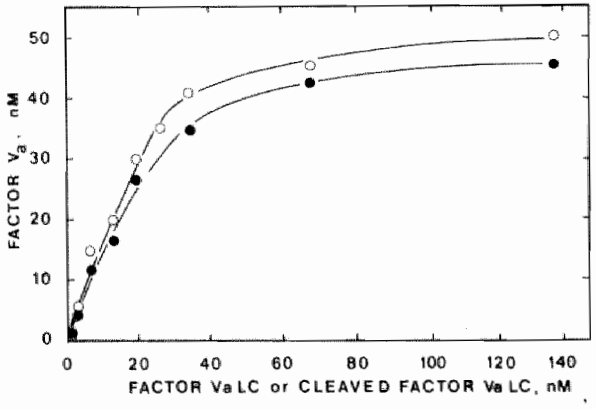

FIGURE 5: Reconstitution of factor Va activity. Varying amounts of activated protein C-cleaved factor Va LC (O) or intact factor Va LC (●) were incubated with factor Va HC (60 nM) in 50 mM Tris, 100 mM NaCl, 10 mM $MnCl_2$, pH 7.5 for 30 min at 37 ^{o}C. Samples were taken and assayed for factor Va activity as described in Materials and Methods.

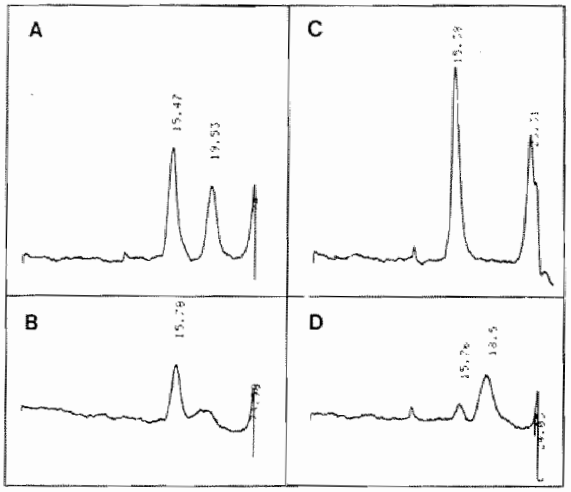

Fig. 6. Size-exclusion HPLC of factor Va subunits and restored factor Va. Elution diagrams for factor Va LC (8 μg) and factor Va HC (10 μg) in the presence of 2 mM EDTA (panel A) or 5 mM $MnCl_2$ (panel B) and for activated protein C-cleaved factor Va LC (8 μg) and factor Va HC (10 μg) in the presence of 2 mM EDTA (panel C) or 5 mM $MnCl_2$ (panel D). AUFS is 0.05. Conditions are as described in Materials and Methods.

Factor Va activity could not be restored by titration of a fixed amount of factor Va LC with unfractionated factor Va HC degradation products, i.e. an equimolar mixture of 72-kDa and 24-kDa fragments (data not shown). To ascertain that the cleaved factor Va HC preparation is not contaminated with activated protein C, we incubated 0.1 μM of factor Va in 20 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.5 with cleaved factor Va HC (50 nM) in the presence of 50 μM phospholipid. Since no decrease in factor Va activity was observed, the presence of activated protein C could be ruled out.

Direct evidence that the factor Va LC degradation products form a complex with factor Va HC was obtained by size-exclusion HPLC. In the presence of EDTA, the elution times of factor Va HC and Va LC were 15.5 min and 19.5 min, respectively (Figure 6A). The 32-kDa and 51-kDa factor Va LC degradation fragments eluted together (elution time was 23.3 min) and close to the salt volume of the column, 24.0 min (Figure 6C). The elution times of factor Va LC and its fragments indicate a nonideal size-exclusion retention mechanism. Apparently, protein-support surface interaction dominates in the chromatogram.

In the presence of Mn^{++}, factor Va HC and factor Va LC form a complex with an elution time of 15.8 min (Figure 6B). Factor Va activity eluted with the protein peak. The elution time of the complex (M_r=174,000) also showed

nonideal size-exclusion behaviour as was observed for factor Va LC.

A mixture of equimolar amounts of factor Va HC and cleaved factor Va LC preincubated in the presence of Mn^{++}, eluted from the column as a major protein peak, which contained factor Va activity. The elution time is 18.5 min. The minor protein peak, elution time of 15.8 min, contained less then 1% of the total recovered factor Va activity (Figure 6D).

DISCUSSION

Single chain bovine factor V (M_r=330,000) is converted by thrombin into factor Va, a two-subunit protein, i.e. a 94-kDa component associated via calcium ions with an 80-kDa component. As a consequence of this action of thrombin, the pro-cofactor acquires factor Xa and prothrombin binding sites (Nesheim et al., 1979; Suzuki et al., 1982) while the phospholipid-binding properties (Van de Waart et al., 1983) are only slightly changed. Because of these interactions, factor Va is essential to the assembly and function of the prothrombinase complex.

However, the structure-function relationship between factor Va subunits and the other components of the prothrombinase complex has not yet been elucidated. Activated protein C has been used to probe the functions of the individual subunits (Walker et al., 1979; Tracy et al., 1983; Suzuki et al., 1983). These studies showed that factor Va activity decreased in parallel with the degradation of the 94-kDa component (factor Va HC).

Information as to the consequences of the cleavage of factor Va LC by activated protein C was not obtained, because the light chain of factor Va was partially (Walker et al., 1979) or not significantly (Suzuki et al., 1983) cleaved. That is, the cleavage of the heavy chain proceeded faster in comparison with the cleavage of the light chain of factor Va. This is in contrast with our findings, where cleavage of the light chain proceeded at the same rate as the cleavage of the heavy chain (Figure 1). It was noted by Walker et al. (1979) that the differences in relative rates of proteolysis of the heavy and light chain disappeared in the presence of phospholipid. In view of the effect of the phospholipid concentration on the pseudo first-order rate constant of inactivation of factor Va and the phospholipid-binding

properties of factor Va and activated protein C (Figure 2), the notion arises that the differences in rates of light chain proteolysis as compared to the rate of proteolysis of the heavy chain might be due to the extent of the solution phase inactivation of factor Va.

Direct evidence that inactivation of factor Va by activated protein C is solely the result of cleavage of the heavy chain was provided by the reconstitution experiments (Figure 5). Whether or not cleavage of the heavy chain results in the loss of factor Xa and/or prothrombin binding to factor Va, is an open question. Preliminary data showed that the 72-kDa fragment, derived from the heavy chain, interacts via calcium ions with phospholipid-bound factor Va LC. Whether or not such a complex binds factor Xa and/or prothrombin is the subject of further investigation.

As to the cleavage of the light chain, it is of interest to see that this proteolytic event does not affect the functional properties of factor Va (Figure 5). We clearly demonstrated that the degradation products of the light chain bind to phospholipid (Figure 4) and form a complex with the heavy chain (Figure 6). It is apparent that both features are essential to the function of factor Va in the prothrombinase complex.

As to the stimulatory effect of phospholipid on the rate of inactivation of factor Va, the two-subunit structure appears to be important. Although the rate of inactivation seems to depend on the amount of phospholipid-bound activated protein C (Figure 2), binding of the substrate to phospholipid is required (Figure 3). Since proteolysis of the subunit that binds to phospholipid by means of the light chain, results in the inactivation of factor Va, conservation of the phospholipid- as well as the heavy chain- binding properties of the degradation products of the light chain is essential to the stimulatoty effect of phospholipids on the inactivation of factor Va by activated protein C.

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CHAPTER VIII

GENERAL DISCUSSION AND SUMMARY

The Central Role of Factor Va in the Prothrombinase Complex.

Under physiological conditions, the blood clotting factor thrombin is generated from its zymogen, prothrombin, by a limited proteolytic action of the prothrombinase complex. This complex consists of the proteolytic enzyme factor Xa, the protein cofactor factor Va, a phospholipid surface provided by blood platelets and calcium ions.

Factor Va, phospholipid and calcium are so-called accessory components. Their effects on the kinetic parameters of prothrombin activation were extensively studied by Rosing and co-workers (1980). They found that phospholipids greatly affect the K_m for prothrombin. Phospholipid decreased the K_m from far above (130 μM) to far below (0.05 μM) the plasma concentration of prothrombin (1.5 μM). Factor Va was found to increase the maximal rate (V_{max}) of thrombin formation about 2000-fold.

In order to explain these findings in mechanistic terms, knowledge about the binding interaction between the different components of the prothrombinase complex (see Figure 1, chapter I) is necessary. Therefore, quantitative binding measurements as described in this thesis, are necessary contribution to a better understanding of the mechanism of prothrombin activation.

Extensive studies (Nelsestuen & Broderius, 1977; Resnick & Nelsestuen, 1980; Mayer & Nelsestuen, 1981) on the binding interaction of prothrombin and factor X(a) with phospholipids have revealed that: 1) the amount of protein bound phospholipid is directly proportional to the phosphatidylserine content of the phospholipid surfaces containing up to 15 mole% of phosphatidylserine, indicating that the proteins causes clustering of the negatively charged phospholipid molecules in the membrane, 2) a chelation model for prothrombin and factor X(a) binding to phospholipid can be proposed where the two interacting species have no net charge; ligands on the protein (γ-carboxyglutamic acid residues) complete the coordination sphere of membrane-bound

calcium and vice versa and 3) the proteins interact almost exclusively to the surface of the phospholipid membrane.

Concerning the binding of factor V(a) to phospholipid it was found that negatively-charged phospholipids are required. In contrast with the calcium requirement for prothrombin- and factor Xa-phospholipid interaction, calcium ions are not involved in factor Va-phospholipid interaction (Bloom et al., 1979). We demonstrated (chapter III) that: 1) increasing calcium concentration or increasing ionic strength inhibit the binding of factor Va to phospholipid containing phosphatidylserine, 2) the binding of factor Va to phospholipid was found to be dependent on the pH when the phospholipid contained phosphatidic acid, 3) phospholipid-bound protein is proportional to phosphatidylserine content (up to 30 mole %) and 4) the phospholipid-binding site of factor V(a) is located on the factor Va light chain (M_r = 80,000), a basic polypeptide with an isoelectric point greater than 9. From these findings, we concluded that there is a direct electrostatic interaction between the phosphate groups of the phospholipids (e.g. phosphatidylserine) and the positively charged amino acid residues of factor V(a).

Since factor Xa, prothrombin and factor Va bind peripherally to negatively-charged phospholipids with dissociation constants of 10^{-7} M, 10^{-7} M and 10^{-8} M, respectively, it is easy to conceive that these proteins compete for binding to the negatively-charged phospholipids. Examples of such competitive binding interactions are presented in chapter V. In the presence of the purified phospholipid-binding fragment of factor Va (factor Va light chain), the dissociation constant of factor Xa- and prothrombin-phospholipid complexes increased with increasing factor Va LC concentration, indicating a competition between factor Va LC and factor Xa or prothrombin for phospholipid binding sites.

However, we observed that in the presence of intact factor Va, i.e. factor Va light chain associated with factor Va heavy chain via calcium ions, the apparent dissociation constants of factor Xa- and prothrombin-phospholipid complexes decreased by three and two orders of magnitude, respectively (chapters II and V). So, in the presence of complete factor Va there is a cooperative binding effect of the proteins. Because this effect is dependent upon both the heavy and the light chain, whereas the heavy chain shows no interactions *per se* with the phospholipid, it can be ruled out that modulation of the membrane structure by the factor Va-phospholipid interaction contributes significantly to the cooperative protein binding. Therefore, it

is unlikely that the formation of an annulus of negatively-charged phospholipids around factor Va, may result in a high affinity site for prothrombin and factor Xa. The increased affinity of factor Xa and prothrombin for phospholipid in the presence of factor Va and vice versa, must rather be due to protein-protein interaction. This coordinate binding interaction between factor Va and factor Xa at a phospholipid surface was extensively studied (chapter II).

The method used to study factor Va-factor Xa interaction is based on the finding that a factor Xa-factor Va complex is the functional enzyme in prothrombin activation (Rosing et al., 1980) and that the rates of prothrombin activation are proportional with the amount of factor Xa-factor Va complex. We demonstrated that in the absence of phospholipid, factor Xa and factor Va formed a stoichiometric (1:1) complex with a dissociation constant of 3×10^{-9} M. The dissociation constant was found to be independent of the prothrombin concentration, indicating that the substrate of the enzymatic complex does not affect the complex formation.

As also reported by Nesheim and co-workers (1979a), we found that the presence of phospholipids greatly decreases the dissociation constant of the factor Xa-factor Va complex. In order to investigate the contribution of the individual protein-phospholipid interactions to the overall binding interaction of the complex at the phospholipid surface, we varied the phospholipid concentration as well as the phosphatidylserine content of the membrane. We observed that for membranes containing a low mole fraction of phosphatidylserine (5%) the apparent dissociation constant of the factor Xa-factor Va complex was about 10-fold lower than the dissociation constant measured without phospholipids. At the same time, as a result of the coordinate binding, a 100- to 1000-fold increase in affinity of each of the individual proteins for the phospholipid surface was measured. This phenomenon is probably of physiological importance. It explains firstly, that even a very poor binding surface is capable of promoting factor Xa- factor Va interaction and secondly, that in the presence of factor Va, the factor Xa-binding capacity of membranes of very low phosphatidylserine content increased greatly. As already pointed out by Rosing and co-workers (1980), the formation of the complete factor Xa-factor Va complex at the phospholipid surface is the only one that can be considered to contribute sufficiently to physiological thrombin formation. The fact that factor Va is capable of

binding factor Xa strongly even if only small amounts of suitable phospholipids are present explains why some investigators (Tracy et al., 1981; Kane & Majerus, 1982) tend to think of the physiological prothrombinase complex at the surface of the not- or incompletely triggered platelet in terms of a specific factor Xa receptor.

From our results, we were not able to distinguish between two different models proposed to explain the action of phospholipids on protein-protein complex formation. These models are: 1) by virtue of the binding of both factor Xa and factor Va to phospholipid vesicles, a dense shell of reactants is produced; the apparent dissociation constant would be related to the density of factor Va and factor Xa at the phospholipid surface (Nesheim et al., 1981) and 2) the apparent increased affinity between factor Xa and factor Va results from additive effects of protein-phospholipid and protein-protein interactions (Nelsestuen, 1978).

If we assume a relative molecular weight of 4×10^6 for sonicated vesicles (Pusey et al., 1982), then we can calculate that, under our experimental conditions the number of vesicles is two to four orders of magnitude higher than the number of factor Xa or factor Va molecules, and therefore the formation of a dense shell of reactants as an important mechanism of membrane function can be precluded.

Apart from its action on factor Xa and prothrombin binding, factor Va also greatly increases the maximal rate of thrombin formation (k_{cat}) by an as yet obscure mechanism. Either in the absence or in the presence of phospholipid, factor Va drastically increases the catalytic efficiency of factor Xa. There is no evidence indicating that factor Va alters the enzymatic properties of factor Xa *per se*. Apparently, the factor Va binding region on prothrombin, i.e. fragment 2, is required in order to observe an effect of factor Va on the catalytic efficiency of factor Xa (Esmon & Jackson, 1974). In addition, Rosing and co-workers (1980) observed that in the absence of factor Va, prethrombin 2 accumulates during the time course of prothrombin activation, while in the presence of factor Va, very low steady state concentrations of prethrombin 2 were measured. Prothrombin activation by factor Xa appears to be an ordered two step reaction where cleavage of the Arg274-Thr275 bond, resulting in the formation of fragment 1.2 and prethrombin 2, is followed by a cleavage in prethrombin 2 resulting in the formation of thrombin (Hibbard et al., 1982). Given the observation that

fragment 1.2 forms a noncovalently-bound complex with prethrombin 2 (Esmon & Jackson, 1974), the notion arises that factor Va is able to prevent the dissociation of prethrombin 2 from the enzymatic complex before it is converted into thrombin; the apparent V_{max} of prothrombin activation would be related to the decreased K_m for prethrombin 2-fragment 1.2 complex. Another explanation for the effect of factor Va on prothrombin activation might be that the interaction between factor Va and prethrombin 2-fragment 1.2 complex presents the substrate, i.e. prethrombin 2, in a manner that it promotes the accessibility of the enzyme, i.e. factor Xa, towards the vulnerable site of the substrate.

Next we wished to investigate was: to what extent factor Va and prothrombin show a direct interaction. As a first attempt, we studied the effect of factor Va on the rotational correlation time of fluorescein-labeled prothrombin (chapter VI). Under conditions where 1.0 μM of factor Va was titrated with prothrombin up to 0.8 μM, no changes in the rotational correlation time of prothrombin were observed, indicating that no complex was formed. However in the presence of phospholipid, factor Va greatly restricted the motion of prothrombin, suggesting that at the phospholipid surface, prothrombin interacts with factor Va.

Direct binding studies of prothrombin to large-volume vesicles in the presence of factor Va showed that phospholipid promotes the stoichiometric (1:1) complex formation between factor Va and prothrombin (chapter V). The apparent dissociation constant of the complex was found to be 4.5 nM and 15.0 nM when the phospholipid contained 20% and 10% phosphatidylserine, respectively. It is apparent that the affinity of factor Va for prothrombin at the phospholipid surface is approximately one order of magnitude lower than the affinity between factor Va and factor Xa at a phospholipid surface. In the presence of high factor Va concentrations, we demonstrated a competitive binding interaction between factor Va and prothrombin at the surface, while factor Va and factor Xa showed a coordinate binding interaction. This indicates that factor Va and prothrombin interact rather weakly as compared to factor Xa-factor Va interaction.

Although the binding interaction between factor Va and prothrombin is relatively weak, the binding that occurs is of paramount significance to the efficiency of prothrombin activation as demonstrated by a 2000-fold increase in k_{cat}, whether or not phospholipids are present. This is a kinetic effect independent of the magnitude of the binding constant.

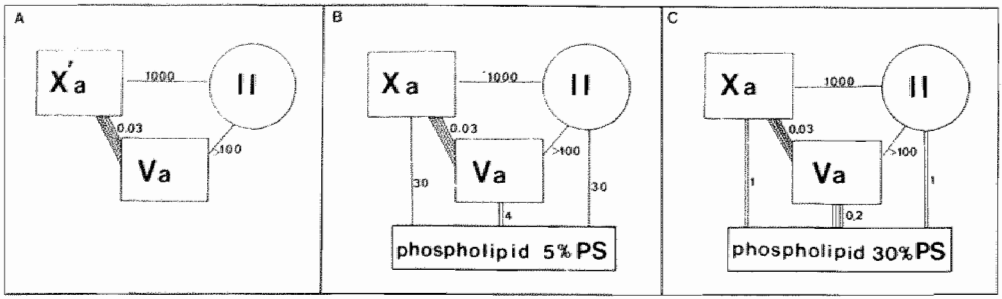

FIGURE 1: Schematic representation of the interactions between the components of the prothrombinase complex. In the absence of phospholipid (A), in the presence of phospholipid containing 5% phosphatidylserine (B), and in the presence of 30% phosphatidylserine (C). The numbers represent the K_d values ($x10^7$ M) as measured in binary systems.

Apart from that, studies utilizing phospholipid vesicles that contained varying amounts of phosphatidylserine showed that in the presence of vesicles that bind factor Xa, factor Va and prothrombin poorly, factor Va greatly decreased the K_m for prothrombin, i.e. under these conditions factor Va has an effect both on prothrombin binding and on k_{cat} (chapter VI). In the presence of vesicles containing 20% DOPS, that already bind prothrombin relatively strongly, factor Va did not significantly affect the K_m for prothrombin.

Figure 1 illustrates our concluding remarks about the central role of factor Va in the prothrombin activation. The prothrombinase complex can be viewed as a dissociable, three-component enzyme (factor Xa, factor Va, phospholipid surface) that binds prothrombin from solution as its substrate. Supporting evidence to this end arose from the following observations: a) no correlation existed between the density of prothrombin at the phospholipid surface and the rate of thrombin formation (Pusey & Nelsestuen, 1983), b) when the vesicles outnumbered the factor Xa and factor Va molecules, a stimulatory effect of phospholipids on factor Xa-factor Va complex formation was observed, that for the function of the prothrombinase complex, the

production of a dense shell of proteins is not an important mechanism of (chapter II) and c) no correlation existed between the density of prothrombin at the phospholipid surface and the K_m for prothrombin when vesicles were used that contained 2-10% phosphatidylserine and factor Va was present, indicating that K_m is a function of the prothrombin concentration in solution (chapter VI).

The dissociation constants (K_d) given in Figure 1 are those measured in binary systems, e.g. factor Va-phospholipid or factor Xa-factor Va. We should realize that the K_d values might change in more complex systems, because the proteins are competing for the same phospholipid binding sites (i.e. phosphatidylserine) and because the K_d depends on the phosphatidylserine content. The actual K_d values in quaternary systems are therefore probably higher than indicated here. As yet, we do not know whether or not the individual protein-phospholipid interactions affect protein-protein interaction, for example by conformational changes. Nevertheless, in our discussion, the K_d values can be used in a qualitative manner.

Based on the K_m for prothrombin in the absence of phospholipid and factor Va, we assume that the dissociation constant of the factor Xa-prothrombin complex is in the order of 100 μM (Rosing et al., 1980). The dissociation constant of the factor Va-prothrombin complex could not be determined. Therefore we propose a minimal value of 10 μM (chapter VI).

The model as presented in Figure 1 suggests that by virtue of the additive free energies of protein-protein plus protein-phospholipid interactions, a tighter binding occurs between substrate and enzyme. If the K_m for prothrombin is related to a binding constant, then it is easy to conceive that under conditions of weak protein-phospholipid affinities (Figure 1B), factor Va provides a tighter binding at the active site because of its phospholipid, factor Xa and prothrombin binding properties. As a result, the K_m for prothrombin would be lowered by factor Va. Obviously, this kinetic contribution of factor Va is masked when the reaction takes place at a phospholipid surface that bind the proteins very well (Figure 1C).

Of course, this is a rather oversimplified view. Other potentially important mechanisms have to be taken into account, e.g. the effect of factor Va on the vectorial organization of prothrombin and factor Xa at the phospholipid surface. The kinetic contribution of such processes is unknown.

We can only speculate on to the relationship between the effect of factor Va on the k_{cat} of thrombin formation and the observed affinity between prothrombin and factor Va. The conversion of prothrombin to thrombin via prethrombin 2 remains as yet unresolved. However, it seems reasonable to assume that the interaction between prothrombin and factor Va is too weak to contribute to an enhanced overall binding at the active site that would result in a lower K_m for prothrombin (prethrombin 2-fragment 1.2). Rather, it is in some manner essential for the increased efficiency of the reaction (Figure 1A).

Regulation of Thrombin Formation at the Level of Factor Va through the Action of Activated Protein C

Activation of factor V by thrombin or the factor V activator from Russell's viper venom results in the exposure of factor Xa and prothrombin binding sites (Nesheim et al., 1979b; Suzuki et al., 1983) and a higher affinity for phospholipids (chapter III).

Because factor Va is a two-subunit molecule that consists of a 94,000 and an 80,000 dalton polypeptide, the question can be addressed whether the subunits have separate and discrete factor Xa, prothrombin or phospholipid binding sites. We demonstrated that the phospholipid binding site is located on the 80,000 dalton fragment (chapter III).

In chapter II, we demonstrated that factor Xa binds neither to the 80,000 dalton nor to the 94,000 dalton fragment of factor Va, indicating that association of the two fragments via Ca^{++} ions is required to obtain factor Xa binding. Whether this is also true for prothrombin binding is not known. However, in chapter V we presented data that indicated that the prothrombin binding site is not located on the 80,000 dalton fragment. We demonstrated that this fragment competes with prothrombin and factor Xa for phospholipid binding sites. The latter observation is in contrast with the findings reported by Tracy and Mann (1983).

Factor Va activity disappears by incubation with activated protein C. We could demonstrate that this inactivation is solely the result of a limited proteolysis of the 94,000 dalton fragment. Whether this results in impaired factor Xa- and/or prothrombin-factor Va interaction is the subject of further investigation. Limited proteolysis of the 80,000 dalton fragment affected

neither the interaction with phospholipid - nor the subunit-subunit interaction (chapter VII).

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SAMENVATTING

De stolling van bloed is het gevolg van een aantal elkaar opvolgende reakties waarin eiwitten (stollingsfaktoren) die in het bloed circuleren geaktiveerd worden. Deze aktiveringen vinden plaats in complexe systemen die bestaan uit een negatief geladen oppervlak (bloedplaatjes membraan) waaraan een enzym en een hulpeiwit of cofaktor zijn gebonden. Dit complex bindt het substraat en zet het om in een aktief enzym. Dit enzym aktiveert vervolgens een ander substraat, enz. De voorlaatste stap in de elkaar opvolgende reakties is de omzetting van prothrombine in thrombine door het prothrombinase complex. Thrombine is het enzym dat fibrinogeen omzet in fibrine dat polimerizeert tot een fibrinenetwerk. In dit prothrombinase complex is faktor Xa het enzym, faktor Va de cofaktor en het oppervlak is de bloedplaatjes membraan. In laboratorium proeven kan deze bloedplaatjes membraan worden vervangen door kunstmatig gemaakte fosfolipide membranen (vesicles). Voor de binding van faktor Xa, faktor Va en prothrombine aan een membraan is het noodzakelijk dat het membraan negatief geladen fosfolipiden bevat.

In dit proefschrift wordt de rol beschreven die faktor Va heeft bij de vorming en samenstelling van het prothrombinase complex. Hiertoe werden de interakties tussen faktor Va en fosfolipiden, faktor Xa en prothrombine bestudeerd.

Een beknopt literatuur overzicht, waarin de opbouw en de belangrijkste eigenschappen van de verschillende componenten van het prothrombinase complex zijn beschreven, is gegeven in hoofdstuk I. Bovendien zijn de belangrijkste studies besproken die hebben bijgedragen tot de kennis van het mechanisme van de prothrombine activering.

Hoofdstuk II beschrijft de zuivering van faktor V uit runderbloed, de aktivering van faktor V, de zuivering van de fragmenten van geaktiveerd faktor V (faktor Va) en de interactie tussen faktor Va en faktor Xa. Voor gezuiverd faktor V werd een molekuul gewicht van 350.000 gevonden. Na aktivering met thrombine worden er 4 fragmenten gevormd, waarvan de fragmenten met molekuulgewicht 94.000 en 80.000 het aktieve faktor Va vormen.

De interaktie tussen faktor Va en faktor Xa werd bestudeerd met een kinetische methode. In vrije oplossing werd een sterke affiniteit tussen faktor Va en faktor Xa gemeten die in aanwezigheid van fosfolipide vesicles

honderd maal wordt versterkt, de repectievelijke dissociatie konstanten zijn 10^{-9} M en 10^{-11} M. De affiniteit in aanwezigheid van fosfolipid vesicles neemt af met afnemend gehalte negatief fosfolipid. Toch zien we dat deze affiniteit van faktor Va en faktor Xa als complex nog steeds 10^{4} maal sterker is in vergelijking met de affiniteiten van faktor Xa en faktor Va afzonderlijk voor het fosfolipide oppervlak. Dit is erg belangrijk voor de snelheid waarmee thrombine gevormd kan worden; het aan fosfolipiden gebonden faktor Xa-Va complex is een veel efficiënter enzym dan faktor Xa alleen. De afzonderlijke faktor Va fragmenten hebben geen meetbare affiniteit voor faktor Xa.

Een directe methode voor het meten van de binding van stolfaktoren aan fosfolipiden is beschreven in hoofdstuk III. De methode maakt gebruik van phospholipide vesicles die in een centrifuge bij 30.000xg kunnen worden neergedraaid. Een eiwit bepaling van de supernatant geeft directe informatie over de hoeveelheid niet-gebonden eiwit. Met behulp van deze methode is aangetoond dat de bindingsplaats voor fosfolipiden op het faktor Va gelokaliseerd is in de subunit met molekuul gewicht 80.000. Dit fragment heeft een netto positieve lading (isoelectrisch punt > 9). De affiniteit van faktor Va hangt af van de concentratie negatieve fosfolipiden, ion sterkte, calcium concentratie en de pH en is dus karakteristiek voor een elektrostatische interaktie.

De invloed van faktor Va op de membraamstruktuur werd bestudeerd in hoofdstuk IV. We vonden dat faktor Va een effect heeft op de "vloeibaarheid" van fosfolipiden boven hun overgangstemperatuur. De veranderingen in de polarisatiegraad van difenylhexatrieen ingebed in de fosfolipiden korreleren met de hoeveelheid faktor Va gebonden aan het oppervlak. Tevens bleek dat binding van faktor Va afhankelijk is van de vloeibaarheid van de membraam.

De eiwit-eiwit interakties in het prothrombinase complex werden met de in hoofdstuk III beschreven, eenvoudige, bindingstechniek bestudeerd (hoofdstuk V). De resultaten laten zien dat onder invloed van faktor Va, de prothrombine binding aan het oppervlak bevorderd wordt. Dit is een direkte aanwijzing voor de vorming van een stoichiometrisch (1:1) complex tussen faktor Va en prothrombine. Het fosfolipide-bindende fragment van faktor Va (faktor Va lichte keten) daarentegen verhinderde de binding van prothrombine aan negatief geladen fosfolipiden. De vorming van het faktor Va-prothrombine complex heeft belangrijke konsekwenties voor de omzetting van prothrombine in thrombine.

Onder invloed van faktor Va wordt de maximale snelheid van thrombine vorming zeer sterk vergroot en indien de reaktie zich afspeeld aan een fosfolipide oppervlak met geringe affiniteit voor prothrombine en faktor Xa, verlaagd faktor Va de K_m voor prothrombine (hoofdstuk VI).

De aktiviteit van het prothrombinase complex wordt gereguleerd door geaktiveerd protein C. Geaktiveerd protein C is in staat faktor Va te inaktiveren. Een onderzoek naar het mechanisme van deze inaktivering is beschreven in hoofdstuk VII. We vonden dat de twee polypeptide ketens van faktor Va beiden gesplitst werden door geaktiveerd protein C. Uit rekonstitutie experimenten bleek dat de abraak van de zware keten van faktor Va gepaard gaat met de inaktivering van faktor Va. Of dit het resultaat is van het elimineren van de prothrombine en/of faktor Xa bindingsplaatsen op het faktor Va, zal verder uitgezocht moeten worden.

In hoofdstuk VIII wordt een algemene beschouwing gegeven van de resultaten van het onderzoek, zoals in dit proefschrift is beschreven.

Nawoord

Allen die op enigerlei wijze hebben bijgedragen aan de tot standkoming van dit proefschrift dank ik daarvoor hartelijk. In het bijzonder dank ik mijn directe medewerkers; Theo Lindhout voor zijn intensieve begeleiding en zijn nimmer aflatende sterk stimulerende inzet. Het is vooral door zijn woord en daad dat de voltooing van dit proefschrift binnen de daarvoor beschikbare tijd kon worden gerealiseerd; Ton Visser voor de samenwerking met betrekking tot de fluorescentie depolarizatie experimenten; Harry Bruls voor de bijdrage die hij op zijn manier, onder zijn moeilijke omstandigheden heeft kunnen leveren, het spijt mij dat hij deze dag niet meer onder ons is; Ben de Kruijff en Jan Rosing voor hun waardevolle discussies en ideeën; Margaret Rand voor het verbeteren van het Engels; Kees Vermeer en Berry Soute voor de vóórbewerkingen van de factor V zuiveringen; Mariet Molenaar voor het typen en verbeteren van de manuscripten; mijn promotor Coen Hemker, de referenten Johan Stefnlo en Wim Hermens voor hun bereidheid zich als zodanig van hun taak te kwijten. José Govers en Jos Kop dank ik behalve voor hun inhoudelijke bijdragen ook voor hun ceremoniële ondersteuning.

Curriculum vitae

De auteur van dit proefschrift werd op 5 september 1948 geboren te Driewegen. Na het basisonderwijs, 4 jaar lager- en 2 jaar middelbaar beroepsonderwijs (L.L.S., L.T.S en M.L.S.), was hij 2 jaar werkzaam op de boerderij van zijn ouders en volgde gedurende die tijd een aantal aan de agrarische sector verwante cursussen. Daarna werkte hij een jaar op een zuivelfabriek als centrifugist en drie jaar als laborant op het laboratorium voor anatomie en embriologie van de Rijksuniversiteit Utrecht. Gedurende die periode volgde hij de part-time opleiding MO-A Natuur- en Scheikunde om vervolgens een jaar werkzaam te zijn als scheikundeleraar aan het Pius X college te Beek (Gld.).

Vanaf 1975 studeerde hij MO-B scheikunde en doctoraal scheikunde met hoofdvak biochemie aan de Rijksuniversiteit te Utrecht, welke studies in juni 1980 met een diploma werden afgesloten.

Van september 1980 tot december 1983 was hij als wetenschappelijk assistent in dienst van de Nederlandse Organizatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O./FUNGO) en werd het in dit proefschrift beschreven onderzoek verricht op de afdeling Biochemie van de Rijksuniversiteit Limburg te Maastricht.

Vanaf maart 1984 zal hij voor 2 jaar werkzaam zijn bij het thrombose- en haemostaseonderzoekslaboratorium van het Hôpital Lariboisière te Parijs.